Metabolism of cruciferous phytoalexins by *Alternaria brassicicola*: metabolites and pathways

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ABSTRACT

Phytoalexins are plant metabolites produced in response to different kinds of stress like pathogen attack, while not present in healthy plants. Cruciferous phytoalexins are produced under pathogen attack and different types of stress by crucifers such as oilseed crops (canola, rapeseed), vegetables (rutabaga, cauliflower, turnip) and condiments (wasabi, mustard). The fungal species *Alternaria brassicicola* (Schwein.) Wiltshire is an economically important pathogen that causes Alternaria black spot in many important crucifer species, namely in *Brassica* species. Although resistance to *A. brassicicola* within the commercially available *Brassica* species is not known, some ecotypes of the wild species *Arabidopsis thaliana* are resistant to this pathogen.

In the first part of this thesis, synthesis of some important cruciferous phytoalexins (camalexin, 1-methylcamalexin, 6-methoxycamalexin, cyclobrassinin, brassilexin, rutalexin and rapalexin A), their antifungal activity and metabolism by the crucifer pathogen *A. brassicicola* was investigated. Almost all tested phytoalexins showed substantial activity against *A. brassicicola* except cyclobrassinin with moderate activity. Metabolic investigation has shown that *A. brassicicola* was able to detoxify camalexin to indole-3-thiocarboxamide (> 5 days), which was transformed further to indole-3-carbonitrile and carboxylic acid, respectively. With the aim of obtaining mechanistic clue on the biotransformation of camalexins, some camalexin derivatives and analogues were synthesized and their antifungal activity as well as metabolism by *A. brassicicola* was investigated. All camalexin derivatives and analogues showed strong antifungal activity against *A. brassicicola*. Metabolic investigation has shown that substituent at C-4′ or C-5′ positions of thiazolyl ring of camalexin stopped oxidative degradation of thiazolyl ring by *A. brassicicola*. In addition, it was obtained that oxidative degradation of the thiazolyl ring requires it to be attached to indole by C-2′. In comparison to camalexins, cyclobrassinin was quickly (ca. 8 hours) detoxified by *A. brassicicola* to S-methyl [(2-sulfanyl-1H-indolyl-3)methyl]carbamothioate, which was further oxidized to sulfinic and sulfonic acid derivatives. Similarly, *A. brassicicola* was able to detoxify brassilexin to 3-aminomethylinindole-2-thione (24 hours). Rutalexin was detoxified by *A. brassicicola* to a highly reactive metabolite that reacted with phomapyrone G, a secondary metabolite produced by *A. brassicicola*, to yield a

stable adduct. All metabolites from biotransformation of mentioned phytoalexins were synthesized and their metabolism as well as antifungal activity against *A. brassicicola* was investigated. It was indicated that *A. brassicicola* can detoxify the cruciferous phytoalexins camalexin, 1-methylcamalexin, 6-methoxycamalexin, cyclobrassinin, brassilexin, and rutalexin. Rapalexin A with strong activity against *A. brassicicola* was resistant to metabolism.

In the second part of this thesis, inhibition of brassinin detoxification by *Leptosphaeria maculans* was investigated. Potential inhibitors were designed and synthesized based on the camalexin scaffold and their inhibitory activity against BOLm was determined using cell-free extracts. Almost all tested compounds showed inhibitory activity against BOLm, however their activity was weaker than camalexin.

ACKNOWLEDGEMENTS

DEDICATION

to

My Mother, Father

and

My wife

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LIST OF ABBREVIATIONS

Ac	acetyl
A. brassicae	*Alternaria brassicae*
A. brassicicola	*Alternaria brassicicola*
A. candida	*Albugo candida*
A. mellea	*Armillaria mellea*
A. oryzae	*Aspergillus oryzae*
A. thaliana	*Arabidopsis thaliana*
Ac_2O	acetic anhydride
AcOH	acetic acid
AIBN	azobisisobutyronitrile
br	broad
B.	*Brassica*
B. bassiana	*Beauveria bassiana*
B. cinerea	*Botrytis cinerea*
BHAb	brassinin hydrolase from *A. brassicicola*
BHLmL2	brassinin hydrolase from *L. maculans* (Laird-2)
BO	brassinin oxidase
Boc	*tert*-butyloxycarbonyl
BOLm	brassinin oxidase from *L. maculans*
BuLi	butyllithium
C cucumerinum	*Cladosporium cucumerinum*
C. polonica	*Ceratocystis polonica*
calcd.	calculated
CHAb	cyclobrassinin hydrolase from *A. brassicicola*
DAD	diode array detector
DBU	1,8-diazabicycloundec-7-ene
DCE	1,2-dichloroethane
DCM	dichloromethane

DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DEA	diethanolamine
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EI	electron ionization (electron impact)
ESI	electrospray ionization
Et	ethyl
Et$_3$N	triethylamine
EtOAc	ethyl acetate
EtOH	ethanol
FCC	flash column chromatography
FTIR	fourier transform infrared spectroscopy
h	hour(s)
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum correlation
HPLC	high performance liquid chromatography
HR	high resolution
HST	host-selective toxin
Hz	hertz
J	coupling constant
L. biglobosa	*Leptosphaeria biglobosa*
L. maculans	*Leptosphaeria maculans*
liq.	liquid
m-CPBA	*meta*-chloroperoxybenzoic acid
M. oryzae	*Magnaporthe oryzae*
m/z	mass/charge ratio
Me	methyl
MeI	methyl iodide
MeOH	methanol

MHz	megahertz
min	minute(s)
MM	minimal media
MS	mass spectroscopy
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
P. chrysogenium	*Penicillium chrysogenium*
PBP	pyridinium bromide perbromide
PCC	pyridinium chlorochromate
PDA	potato dextrose agar
Phe	phenylalanine
PMS	phenazine methosulfate
ppm	part per million
PPTS	pyridinium *p*-toluenesulfonate
PTLC	preparative thin layer chromatography
Pyr	pyridine
R. solani	*Rhizoctonia solani*
r.t.	room temperature
satd. sol.	saturated solution
SsBGT1	recombinant brassinin glucosyltransferase of *S. sclerotiorum*
S. sclerotiorum	*Sclerotinia sclerotiorum*
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TMSCl	trimethylsilyl chloride
TTFA	thallium (III) trifluoroacetate
t_R	retention time
Tyr	tyrosine
UV	ultraviolet
V	volume

1 INTRODUCTION

1.1 General objectives

Phytoalexins are antimicrobial plant secondary metabolites elicited by biotic (microbial attacks) and abiotic (UV radiation or heavy metal salts) factors and are not usually produced in healthy plants (Pedras et al., 2011b; VanEtten et al., 1994). Fungal pathogens are able to metabolize phytoalexins to less or non-toxic compounds through enzyme-catalyzed reactions (Pedras et al., 2011b; Pedras and Ahiahonu, 2005). Inhibition of these fungal detoxifying enzymes could stop cruciferous pathogens from invading crucifers (Pedras and Minic, 2014; Pedras et al., 2012; 2009b). Detoxification of several cruciferous phytoalexins by plant pathogens was investigated and their transformation pathways were determined (Pedras, 2014; Pedras et al., 2011b). Some cruciferous phytoalexins were shown to be resistant to metabolism by plant pathogens (Pedras et al., 2011b). Phytoalexins that are resistant to metabolism are of interest to engineer plants with higher disease resistance levels. Toward this goal, it is important to study the potential metabolism of these compounds and derivatives by plant pathogenic fungi.

The specific objectives of my PhD work are to:

o Investigate the metabolism and determine the products of transformation of the cruciferous phytoalexins camalexin (**1**), 1-methylcamalexin (**2**), 6-methoxycamalexin (**3**), cyclobrassinin (**4**), rutalexin (**5**), brassilexin (**7**), and rapalexin A (**8**) by *Alternaria brassicicola;*

o Investigate the metabolism and determine the products of transformation of the camalexin related structures, 1-methylcyclobrassinin (**193**) and 1-methylbrassilexin (**165**) by *Alternaria brassicicola;*

o Determine the antifungal activity of phytoalexins, selected compounds and metabolites resulting from biotransformation against *Alternaria brassicicola;*

o Determine the effect of camalexin related structures on the rate of brassinin detoxification by *Leptosphaeria maculans* using cell-free extracts.

1.2 Crucifers and fungal pathogens

Crucifers (family Brassicaceae, syn. Cruciferae) are important sources of edible and industrial oils, condiments and forage. Chinese cabbage (*Brassica campestris* var. *Pekinensis*), rutabaga (*Brassica napus* var. *napobrassica*), turnip (*B. campestris* var. *rapifera*), kale (*Brassica oleracea* var. *acephala*), cabbage (*B. oleracea* var. *capitata*), cauliflower (*B. oleracea* var. *italica*) and many more known vegetables are crucifers (Gomez-Campo, 1999). Crucifers contain some important model plants. For example, *Arabidopsis thaliana* (Thale cress) has been established as an important model plant, and was the first flowering plant to have its genome sequenced (Theologis et al., 2000).

1.2.1 Secondary metabolites of crucifers

Organic compounds produced by plants are either primary or secondary metabolites. Primary metabolites such as sugars, amino acids, common fatty acids, and nucleotides are essential for basic metabolic processes of the plants. In contrast, secondary metabolites are not essential for basic plant growth and development (Hartmann, 2007). Secondary metabolites participate in defense mechanisms against stress and play important roles in the fitness of their producers (Bednarek and Osbourn, 2009; Bennett and Wallsgrove, 1994). Crucifers synthesize and accumulate a variety of biologically active secondary metabolites, such as phytoalexins and phytoanticipins (Bednarek, 2012; Pedras et al., 2011b).

1.2.1.1 Phytoalexins

Phytoalexins are antimicrobial plant metabolites elicited by biotic (microbial attacks) and abiotic (UV radiation or heavy metal salts) factors and are not usually produced in healthy plants (Pedras et al., 2011b; VanEtten et al., 1994). Until now 53 phytoalexins (indolyl and non indolyl) have been isolated and characterized from stressed crucifers; however, only a limited number of crucifer species have been investigated. The majority of the cruciferous phytoalexins were isolated from the *Brassica* species (Pedras et al., 2011b; 2015; Pedras and To, 2015). Recent

comprehensive reviews dealing with indolyl cruciferous phytoalexins have been published (Pedras et al., 2011b; Pedras et al., 2000). For this reason, only reports of immediate interest to work described in this thesis as well as the non-indolyl cruciferous phytoalexins are reviewed.

Indolyl phytoalexins

Camalexin (**1**) was first detected and isolated from *Camelina sativa* (false flax) infected with *Alternaria brassicae* (Ayer et al., 1992) and then was isolated from the model plants *A. thaliana* infected with *Pseudomonas syringae* (Glawischnig, 2007; Pedras et al., 2011b; Tsuji et al., 1992). The structure of camalexin (**1**) was confirmed by its synthesis in 1992 (Ayer et al., 1992; Pedras et al., 2011b). Camalexin (**1**) showed strong antifungal activity against a variety of cruciferous pathogens such as *Alternaria brassicicola* (Schwein.) Wiltshire, *Alternaria brassicae* (Berk.) Sacc, *Leptosphaeria maculans* (Desm.) Ces. et de Not. (asexual stage *Phoma lingam* (Tode ex Fr.) Desm.), *Botrytis cinerea* Pers. Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel), *Rhizoctonia solani* Kuhn (Pedras et al., 2011b). Furthermore, camalexin (**1**) was shown to be important in the resistance of *A. thaliana* (Columbia) to *A. brassicicola*; that is, mutants of *A. thaliana* deficient in camalexin (**1**) production were highly susceptible to *A. brassicicola* (Thomma et al., 1999). Selected structures of indolyl phytoalexins produced by crucifers are shown in **Figure 1.1**; the phytoalexin structures relevant to work described in this thesis are grouped in the rectangular box.
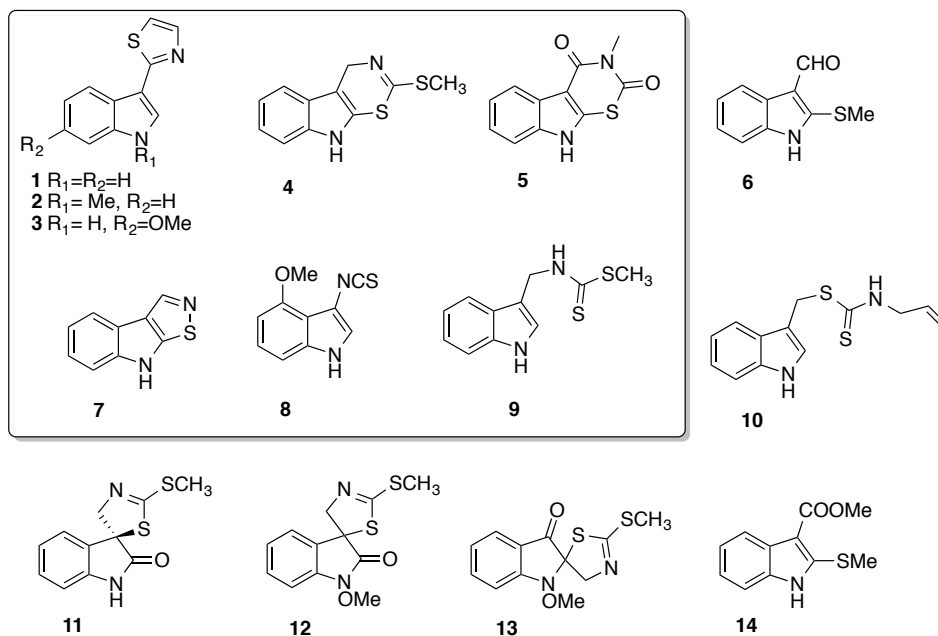
Figure 1.1 Structures of selected indolyl cruciferous phytoalexins: camalexin (**1**), 1-methylcamalexin (**2**), 6-methoxycamalexin (**3**), cyclobrassinin (**4**), rutalexin (**5**), brassicanal A (**6**), brassilexin (**7**), rapalexin A (**8**), brassinin (**9**), brussalexin (**10**) spirobrassinin (**11**), 1-methoxyspirobrassinin (**12**), erucalexin (**13**), brassicanate A (**14**), (Pedras et al., 2011b).

Cyclobrassinin (**4**) was first isolated from Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) heads inoculated with bacterium *Pseudomonas cichorii* in 1986 and its synthesis confirmed the proposed structure (Pedras, 2014; Pedras et al., 2011b; Takasugi et al., 1986). Cyclobrassinin (**4**) is a biosynthetic precursor of rutalexin (**5**) and brassilexin (**7**) (Pedras, 2014; Pedras et al., 2011b). Cyclobrassinin (**4**) showed antifungal activity against a broad range of pathogens such as, *A. brassicae*, *B. cinerea*, *C. cucumerinum*, *L. maculans*, *R. solani* and *Sclerotinia sclerotiorum* (Lib.) de Bary (Pedras, 2014). Cyclobrassinin (**4**) was synthesized in 35% yield from brassinin (**9**), which, in turn, was synthesized from aldehyde **26** in 58% overall yield (**Scheme 1.1**) (Pedras et al., 2011b; Takasugi et al., 1986).
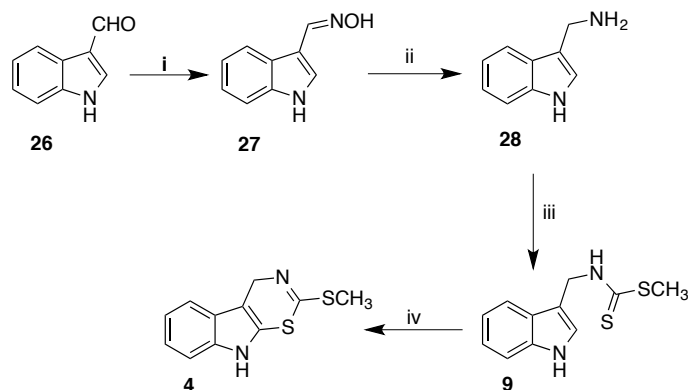
Scheme 1.1 Synthesis of cyclobrassinin (**4**). Reagents and conditions: (i) NH$_2$OH.HCl, Na$_2$CO$_3$, 1 h, 80 °C; (ii) NiCl$_2$.6H$_2$O, NaBH$_4$, MeOH, 10 min, 0 °C; (iii) Et$_3$N, pyridine, CS$_2$, 10 min, 0 °C, MeI, 30 min, 0 °C; (iv) Pyridinium bromide perbromide (PBP), 1,8-diazabicycloundec-7-ene (DBU), THF, 100 min, r.t., 35% (Takasugi et al., 1986).

Rutalexin (**5**) was first isolated and characterized from stressed (UV light) rutabaga (*Brassica napus* L. ssp. *rapifera*) tubers (Pedras et al., 2004b). Synthesis of rutalexin (**5**) confirmed the proposed structure (**Scheme 1.2**) (Pedras et al., 2004b). The synthesis started with oxidation of aldehyde **15** to *N*-Boc-2-chloroindole-3-carboxylic acid (**16**). Acid **16** was reacted with thionyl chloride followed by methylamine to provide amide **17**. Amide **17** was reacted with NaSH in DMF/H$_2$O to yield sulfanylamide **18**. Finally, rutalexin (**5**) was obtained from reaction of sulfanylamide **18** with phosgene followed by subsequent deprotection at 165–170 °C in 24% overall yield (**Scheme 1.2**) (Pedras et al., 2004b).
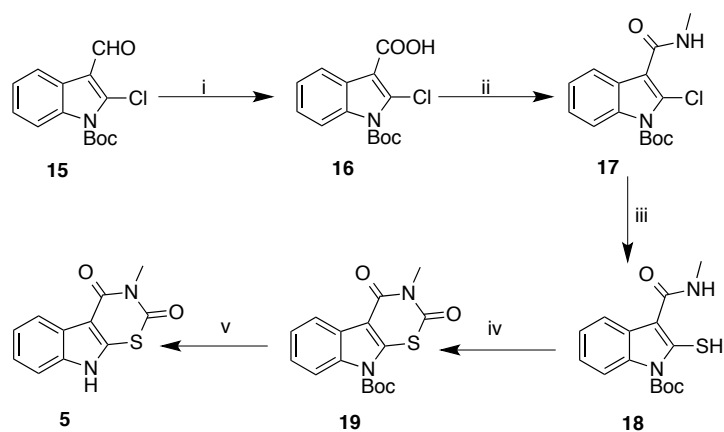
Scheme 1.2 Synthesis of rutalexin (**5**). Reagents and conditions: (i) NaClO$_2$, 2-methylbut-2-ene, KH$_2$PO$_4$, *tert*-butyl alcohol/H$_2$O, r.t., 2 h, 96%; (ii) SOCl$_2$, THF, rt, 3 h, then CH$_3$NH$_2$, THF, 0 °C, 20 min, 85% (based on acid **16**); (iii) NaSH, DMF/H$_2$O, 0 °C, 2 h; (iv) NaH, THF, 0 °C, 10 min then COCl$_2$ (20% in toluene), -78 °C to rt, 4 h, 30% (based on **17**); (v) 165–170 °C, 30 min, 100% (Pedras et al., 2004b).

Rutalexin (**5**) was also prepared from 9-Boc-2-methoxy-4-oxo-[1,3]thiazino[6,5-*b*]indole (**20**) and cyclobrassinin (**4**) (Budovská et al., 2015). 9-Boc-1,3-thiazino[6,5-*b*]indole-2,4-dione (**21**) was synthesized from hydrolysis of **20** (HCl) and then reacted with MeI in presence of DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) to yield *N*-Boc-rutalexin (**19**). As in the previous synthesis, deprotection of compound **19** under solvent free conditions yielded rutalexin (**5**) in 76% overall yield (**Scheme 1.3**) (Budovská et al., 2015).
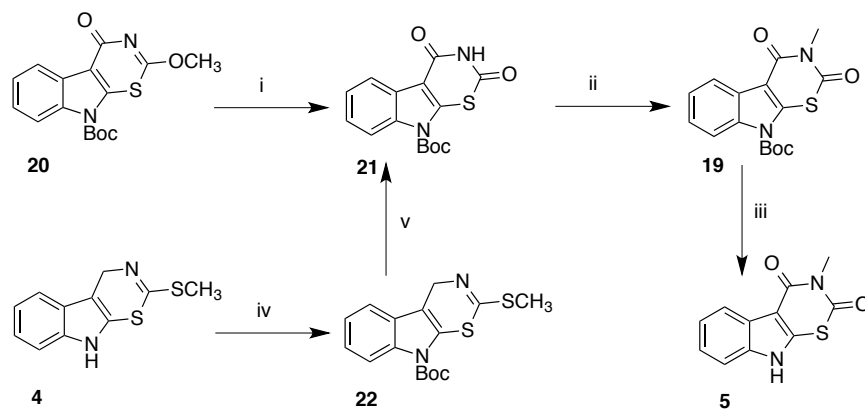
Scheme 1.3 Synthesis of rutalexin (**5**). Reagents and conditions: (i) HCl/H$_2$O (1:1, cat.), acetone, r.t., overnight, 80%; (ii) DBU, CH$_3$I, THF, under N$_2$, r.t., 2 h, 95%; (iii) 165–170 °C, 30 min, quant.; (iv) Boc-anhydride, DMAP, THF, 5 °C, 1 h, 88%; (v) PCC, CH$_2$Cl$_2$, 24 h, 66% (Budovská et al., 2015).

Synthesis of compound **21** was also reported from oxidation of *N*-Boc-cyclobrassinin (**22**) using pyridinium chlorochromate (PCC) in 66% yield (**Scheme 1.3**). Although these routes yielded rutalexin (**5**) in reasonable overall yield, from either 9-tert-butoxycarbonyl-2-methoxy-4-oxo-[1,3]thiazino[6,5-*b*]indole (**20**) or *N*-Boc-cyclobrassinin (**22**), compounds **20** or **22** were obtained from multi step reactions in low overall yield (Suchy et al., 2001). For example, *N*-Boc-cyclobrassinone (**20**) was synthesized from aldehyde **23** in 17% overall yield (**Scheme 1.4**) (Kutschy et al., 2002).
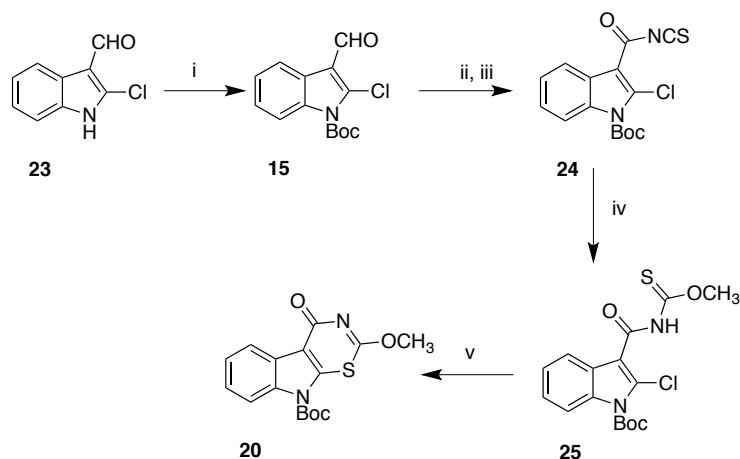
Scheme 1.4 Synthesis of *N*-Boc-cyclobrassinone (**20**). Reagents and conditions: (i) Boc$_2$O, DMAP, THF, 5°C, 1 h, 68%; (ii) NBS, AIBN, tetrachloromethane, reflux, 10 min; (iii) KSCN, acetone, r.t., 15 min, 41% (based on **15**); (iv) CH$_3$OH, acetone, r.t., 2 h; (v) Et$_3$N, r.t., 1 h, 61% (yield is based on isothiocyanate **24**).

Due to the low solubility of rutalexin (**5**) in PDA, its antifungal activity was determined using a TLC bioassay: rutalexin (**5**) inhibited completely the growth of *C. cucumerinum* at 2 × 10^{-6} mol (Pedras et al., 2004b).

Brassilexin (**7**) was isolated and characterized in 1988 from the leaves of mustard (*Brassica juncea*) (Devys et al., 1988; Pedras et al., 2011b). The proposed structure of brassilexin (**7**) was confirmed with synthesis two years later (Devys and Barbier, 1990). Brassilexin (**7**) showed strong antifungal activity against *L. maculans*, *A. brassicae*, *R. solani* and *S. sclerotiorum* (Pedras, 2014).

Rapalexin A (**8**) was the first naturally occurring aromatic isothiocyanate. Rapalexin A (**8**) was isolated from canola leaves (*Brassica rapa*), infected with *Albugo candida*, Pers. ex Chev., Kuntze. Its structure was confirmed by synthesis (Pedras et al., 2011b; 2007b) (**Scheme 1.5**). Nitration of 4-methoxyindole (**31**) using AgNO$_3$ yielded 4-methoxy-3-nitroindole (**30**) in 30% yield. Standard hydrogenation of nitro group of **30** followed by reaction with thiophosgene afforded rapalexin A (**8**) (**Scheme 1.5**) (Pedras et al., 2011b; 2007b). Later on 4-methoxy-3-nitroindole (**30**) was synthesized from methoxylation of 3-nitroindole (**29**) using thallium (III) trifluoroacetate (TTFA) in 64% yield (Pedras and Yaya, 2012). Rapalexin A (**8**) showed strong

antifungal activity against *L. maculans* (Pedras and Sarma-Mamillapalle, 2012) and *A. candida* (Pedras et al., 2011b).
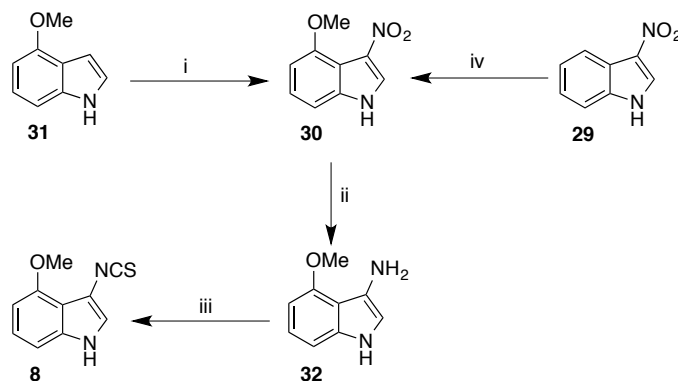
Scheme 1.5   Synthesis of rapalexin A (**8**). Reagents and conditions: (i) AgNO$_3$, benzoyl chloride, CH$_3$CN, 30%; (ii) Pd/C, H$_2$, AcOH; (iii) CH$_2$Cl$_2$, CaCO$_3$, CSCl$_2$, 20% over two steps; (Pedras et al., 2007b); (iv) TFA, TTFA, I$_2$, CuI, DMF, NaOMe, MeOH, 64%, (Pedras and Yaya, 2012).

Brassinin (**9**), the first phytoalexin reported from Brassicaceae, was first isolated and characterized from stressed Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) by Takasugi and co-workers (Pedras et al., 2011b; Takasugi et al., 1986). The structure of brassinin (**9**) was confirmed with its synthesis from indole-3-methanamine (**28**) (**Scheme 1.1**) (Takasugi et al., 1986). Brassinin (**9**) is a biosynthetic precursor of several cruciferous phytoalexins (**Scheme 1.6**) (Pedras et al., 2011b). Furthermore, brassinin (**9**) showed strong antifungal activity against cruciferous pathogens such as, *L. maculans*, *S. sclerotiorum* and *B. cinerea* (Pedras et al., 2011b).

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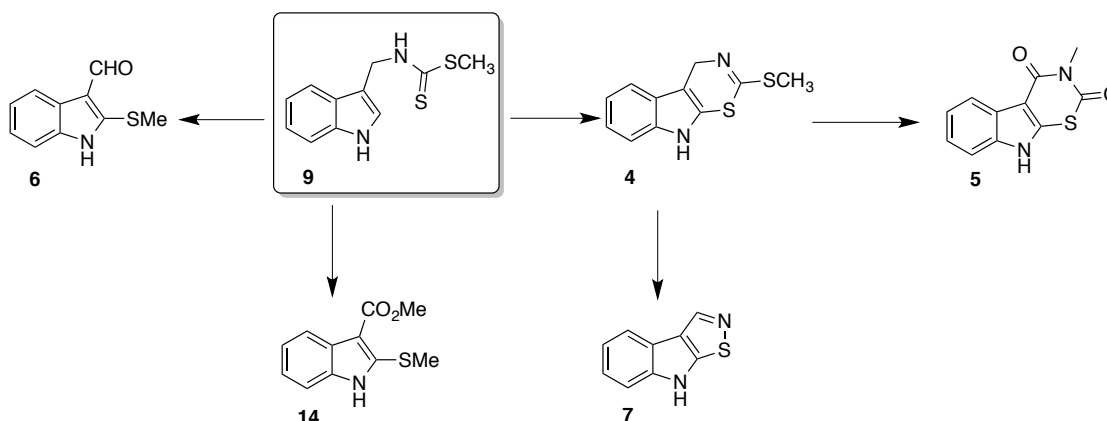
Scheme 1.6 Biosynthetic relationship among brassinin (**9**) and other phytoalexins cyclobrassinin (**4**), rutalexin (**5**), brassicanal A (**6**), brassilexin (**7**), and brassicanate A (**14**) (Pedras et al., 2011b).

Non-indolyl phytoalexins

The hypothesis that crucifer species could biosynthesize non-indolyl phytoalexins was formulated long time ago when 4-hydroxybenzylisothiocyanate (**33**) was isolated as a major antifungal component from white mustard (*Sinapis alba*) sprayed with a copper(II) chloride (CuCl$_2$) solution (Pedras and Smith, 1997).
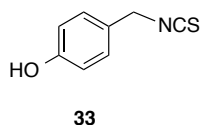
Figure 1.2 Structure of 4-hydroxybenzylisothiocyanate (**33**).

Eighteen years later, the first group of non-indolyl cruciferous phytoalexins was isolated and characterized from the copper(II) chloride treated leaves of watercress (*Nasturtium officinale* R. Br.) (Pedras and To, 2015). Nasturlexin A (**34**), nasturlexin B (**35**) and tridentatol C (**36**) are the first three non-indolyl cruciferous phytoalexins containing a phenyl ring instead of indole in their structures (**Figure 1.3**).
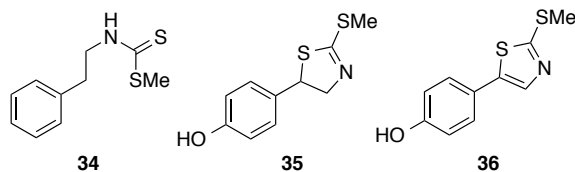
Figure 1.3   Structures of non-indolyl cruciferous phytoalexins; nasturlexin A (**34**), nasturlexin B (**35**), tridentatol C (**36**) (Pedras and To, 2015).

Phytoalexins from *Barbarea* species, winter cress (*Barbarea vulgaris* R. Br) and upland cress (*Barbarea verna* (P. Mill.) Aschers) were isolated from the leaves sprayed with copper(II) chloride (Pedras et al., 2015). The new non-indolyl phytoalexins, nasturlexin C (**37**), nasturlexin C sulfoxide (**38**), nasturlexin D (**39**), nasturlexin D sulfoxide (**40**) were isolated from both species (**Figure 1.4**) (Pedras et al., 2015).
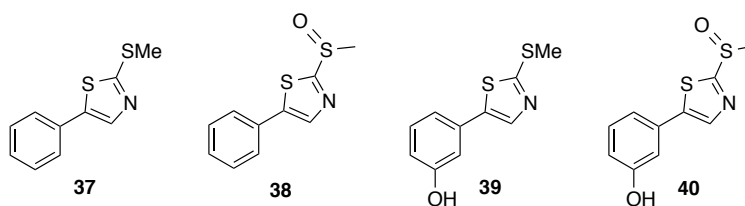
Figure 1.4   Structures of nasturlexin C (**37**), nasturlexin C sulfoxide (**38**), nasturlexin D (**39**), nasturlexin D sulfoxide (**40**) (Pedras et al., 2015).

Tridentatol C (**36**) is a secondary metabolite previously isolated from *Tridentata marginata* in 1996 (Lindquist et al., 1996). The structure of tridentatol C (**36**) was first confirmed by X-ray crystallography (Lindquist et al., 1996) and later, synthesized (Jayatilake and Baker, 1999). Tridentatol C (**36**) was synthesized from (±)-octopamine (**41**) *via* nasturlexin B (**35**). (±)-Octopamine (**41**) was reacted with CS$_2$/MeI in the presence of Et$_3$N to yield nasturlexin B (**35**) in 90% yield. Nasturlexin B (**35**) was then oxidized to tridentatol C (**36**) using DDQ in 1,4-dioxane in 70% yield (**Scheme 1.7**) (Jayatilake and Baker, 1999; Pedras and To, 2015).
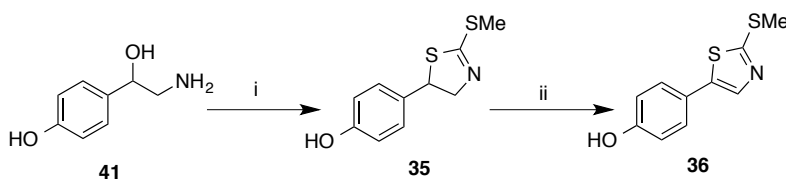
Scheme 1.7 Syntheses of nasturlexin B (**35**) and tridentatol C (**36**). Reagents and conditions: (i) CS_2, MeI, Et_3N, DCE, reflux, 90%; (ii) DDQ, 1,4-dioxane, reflux, 70% (Jayatilake and Baker, 1999; Pedras and To, 2015).

Nasturlexin C (**37**) was synthesized from 2-amino-1-phenylethanol hydrochloride (**42**) in 20% overall yield. Compound **42** was chlorinated upon reaction with $SOCl_2$ and the resulting intermediate was treated with CS_2 that cyclized spontaneously. Methylation with MeI afforded compound **43**, which was oxidized to nasturlexin C (**37**) with DDQ in 1,4-dioxane (Pedras et al., 2015).
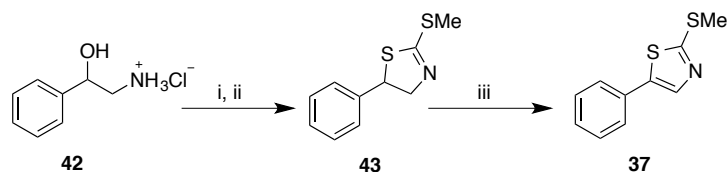
Scheme 1.8 Synthesis of nasturlexin C (**37**). Reagents and conditions: (i) $SOCl_2$, DMF, $CHCl_3$, 0 °C; (ii) CS_2, pyridine, Et_3N, MeI, r.t., 29% (over 2 steps); (iii) DDQ, 1,4-dioxane, 100 °C, 70% (Pedras et al., 2015).

The sulfoxide derivatives of nasturlexin C and D, **38** and **40**, were obtained by *m*-CPBA oxidation of nasturlexin C (**37**) and nasturlexin D (**39**). The sulfoxide **40** was obtained in quantitative yield using two equivalents of *m*-CPBA. Similarly, nasturlexin C (**37**) was oxidized to sulfoxide **38** (Pedras et al., 2015).
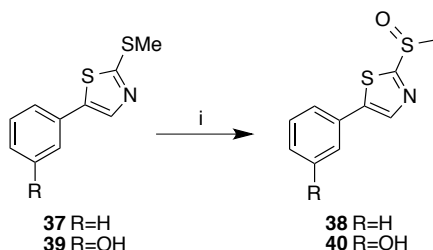
Scheme 1.9 Syntheses of nasturlexin C sulfoxide (**38**) and nasturlexin D sulfoxide (**40**). Reagent and condition: (i) *m*-CPBA (1eq for **38** and 2eq for **40**), CH_2Cl_2, 72% (**38**) and quantitative (**40**) (Pedras et al., 2015).

Nasturlexin A (**34**) was synthesized from phenylethylamine (**44**) after treatment with CS_2 and MeI (**Scheme 1.10**) (Gaspari et al., 2006; Pedras and To, 2015).
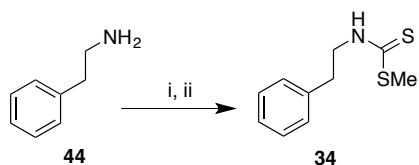
Scheme 1.10 Synthesis of nasturlexin A (**34**). Reagents and conditions: (i) CS_2, Et_3N, pyridine, 15 min, r.t., (ii) MeI, 15 min, 43% (over two steps)(Pedras and To, 2015).

The antifungal activities of nasturlexin A (**34**), nasturlexin B (**35**), nasturlexin C (**37**), nasturlexin D (**39**), nasturlexin C sulfoxide (**38**), nasturlexin D sulfoxide (**40**), and tridentatol C (**36**) were determined against the fungal pathogens *A. brassicicola*, *L. maculans* and *S. sclerotiorum* (Pedras et al., 2015; Pedras and To, 2015). Except for sulfoxides **38** and **40** that were not active against *S. sclerotiorum*, all tested compounds displayed strong inhibitory activity (Pedras et al., 2015; Pedras and To, 2015). The biosynthetic pathway of non-indolyl phytoalexins was proposed to derive from tyrosine (Tyr) or phenylalanine (Phe) (**Scheme 1.11**) (Pedras and To, 2015). **Scheme 1.11** summarizes the proposed biosynthetic pathway of these non-indolyl cruciferous phytoalexins (Pedras et al., 2015; Pedras and To, 2015).

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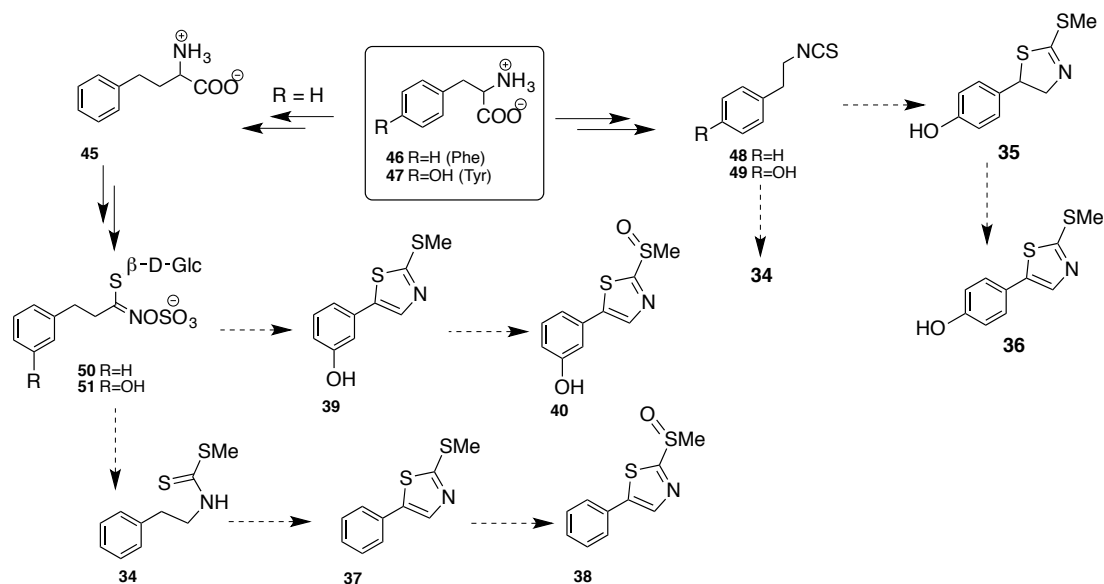
Scheme 1.11 Proposed biosynthetic relationships (dashed arrows) among phenylalanine (Phe) (**46**), tyrosine (Tyr) (**47**) and non-indolyl cruciferous phytoalexins (Pedras et al., 2015; Pedras and To, 2015).

1.2.1.2 *Phytoanticipins*

Phytoanticipins are antimicrobial plant secondary metabolites that are present in healthy plants and their concentrations may increase with stress (VanEtten et al., 1994). Selected structures of phytoanticipins produced by crucifers are shown in **Figure 1.5**. The distinction between phytoalexins and phytoanticipins is not always obvious. Some compounds are phytoalexins in one species and phytoanticipins in another. For example, methyl-1-methoxyindole-3-carboxylate (**55**) was reported as a phytoanticipin from *A. thaliana* while the same compound isolated from stressed wasabi was a phytoalexin (Pedras and Adio, 2008; Pedras et al., 1999). Arvelexin (**54**) a phytoalexin isolated from the wild crucifer *Thlaspi arvense* is a phytoanticipin in canola (*brassica napus* L. spp. oleifera) infected by *Plasmodiophora brassicae* (clubroot) (Pedras et al., 2003; 2008b).
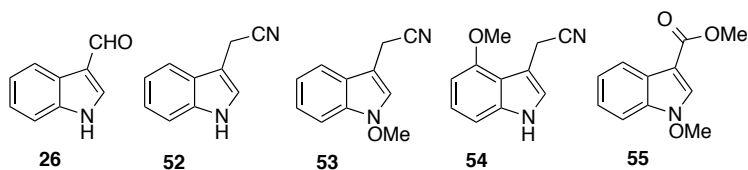
Figure 1.5 Structures of phytoanticipins: Indole-3-carboxaldehyde (**26**), indolyl-3-acetonitrile (**52**), 1-methoxyindolyl-3-acetonitrile (**53**), 4-methoxyindolyl-3-acetonitrile (**54**, i.e. arvelexin), and methyl 1-methoxyindole-3-carboxylate (**55**).

1.2.2 Fungal pathogens, Alternaria species

Cruciferous plants (*Brassicaceae*) are susceptible to *Alternaria* species. *Alternaria* species such as *A. brassicae*, *A. brassicicola*, *A. raphani*, and *A. alternata* cause major damage on *Brassicae* plants (Nowicki et al., 2012). Alternaria black spot, the most common disease of *Brassica* species, is caused by *A. brassicicola*, in conjunction with *A. brassicae* (Pedras et al., 2009a). Alternaria black spot symptoms appear on almost all parts of the host plants as necrotic lesions.

Some secondary metabolites produced by *Alternaria* species are toxic to plants (phytotoxins) and cause cell death at low concentration (Thomma, 2003). Phytotoxins facilitate fungal colonization of plants (Walton, 1996). Brassicicolin A (**56**) is the only phytotoxin produced by *A. brassicicola* (Pedras et al., 2009a). Secondary metabolites from *Alternaria* species showed a variety of biological activities for example, destruxins from *A. brassicae* showed antitumor, antiviral and insecticidal activities (Liu and Tzeng, 2012). Secondary metabolites from *Alternaria* fungi and their bioactivities were reviewed recently by Lou and co-workers (Lou et al., 2013). Herein, work of immediate interest in this thesis is reviewed.

1.2.2.1 *Secondary metabolites of Alternaria brassicicola*

Alternaria brassicicola produces several secondary metabolites including nitrogen-containing metabolites, terpenoids, pyranones and some other metabolites (Lou et al., 2013). Brassicicolin A (**56**) was the first metabolite isolated from *A. brassicicola* (Ciegler and Lindenfelser, 1969). Twenty years after isolation of brassicicolin A (**56**), its structure was established as a mixture of epimers (Gloer et al., 1988). Later on brassicicolin A (**56**) was

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isolated from liquid cultures of *A. brassicicola* and established to be a host-selective toxin (HST) (Pedras et al., 2009a).
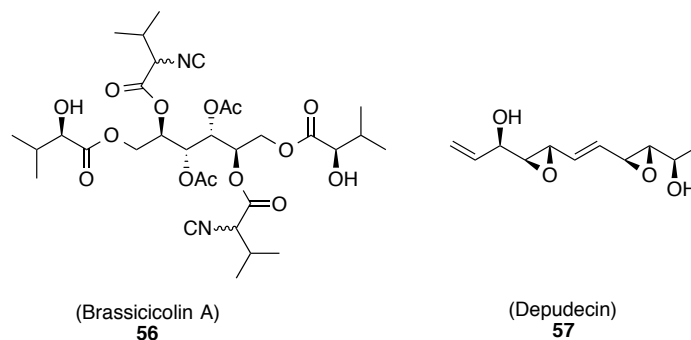
Figure 1.6   Structures of brassicicolin A (**56**) and depudecin (**57**).

Phytotoxicity of brassicicolin A (**56**) was determined on leaves of *Brassica juncea* cv. Cutlass (susceptible), *Brassica napus* cv. Westar (tolerant) and *Sinapis alba* cv. Ochre (resistant) (Pedras et al., 2009a). Results of phytotoxicity experiments indicated that brassicicolin A (**56**) was a HST causing damage only on leaves of *B. juncea* and *B. napus* (Pedras et al., 2009a). Depudecin (**57**) was isolated from cultures of *A. brassicicola* grown in minimal media (MM) in 1992 (Matsumoto et al., 1992). Depudecin (**57**) was reported as a histone deacetylase inhibitor (Kwon et al., 2003; 1998). Depudecin-minus mutants of *A. brassicicola* revealed that depudecin plays a minor role in the virulence of *A. brassicicola* on cabbage (Wight et al., 2009). Depudecin (**57**) was chemically synthesized from tetraol **58** in 1995 (Shimada et al., 1995). Tetraol **58** was treated with $MeC(OMe)_3$ and catalytic amount of pyridinium *p*-toluenesulfonate (PPTS) in presence of trimethylsilyl chloride and triethylamine to yield diacetoxy dichloride **59**. Basic hydrolysis of **59** followed by spontaneous cyclization yielded bis-*trans* epoxide **60** in 74% overall yield. Finally, deprotection using mercuric chloride and excess calcium carbonate yielded depudecin (**57**) (**Scheme 1.12**) (Shimada et al., 1995).
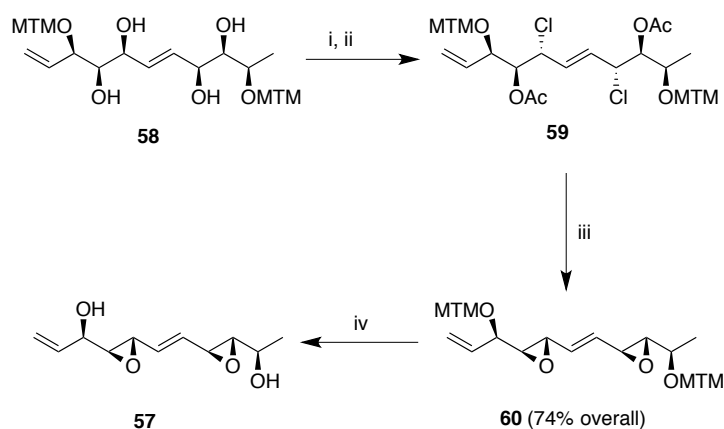
Scheme 1.12 Synthesis of depudecin (**57**). Reagents and conditions, (i) MeC(OMe)₃, cat. PPTS, 23 °C; (ii) trimethylsilyl chloride (TMSCl), Et₃N, 23 °C; (iii) K₂CO₃, MeOH, 23 °C; (iv) 50 eq. HgCl₂-CaCO₃, MeCN-H₂O, 23 °C, 3.5 h, 52% (Shimada et al., 1995).

In 1999 six potentially phytotoxic compounds were isolated from liquid cultures of *A. brassicicola* that were called brassicicenes A-F (**61-66**), however due to the small amounts obtained from culture extracts, their phytotoxicity was not determined (MacKinnon et al., 1999). Later on brassicicene G (**67**), brassicicene H (**68**), brassicicene I (**69**) (Pedras et al., 2009a), brassicicene J (**70**) and brassicicene K (**71**) (Kenmoku et al., 2014) were also isolated and characterized from cultures of *A. brassicicola*.
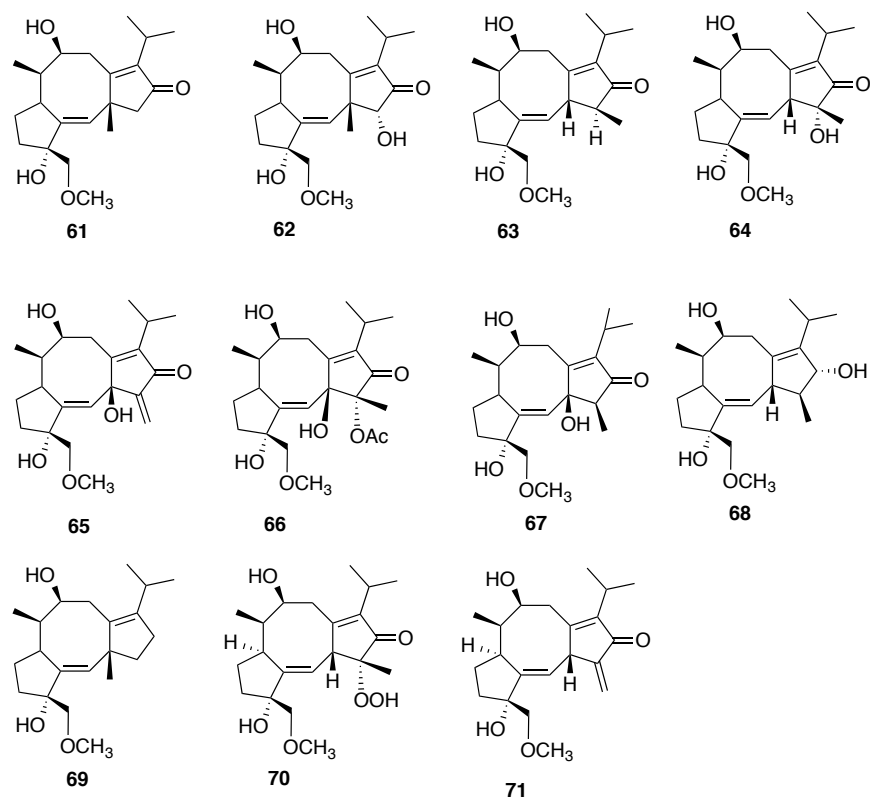
Figure 1.7 Structures of brassicicene A-F (**61-66**), brassicicene G-I (**67-69**) and brassicicene J-K (**70** and **71**).

Phomapyrones A, F and G (**72, 74** and **75**) and infectopyrone (**73**) were isolated from the non-phytotoxic fractions of broth extracts of cultures of *A. brassicicola* (Pedras et al., 2009a); these metabolites were reported previously from *L. maculans* and *L. biglobosa* (Pedras and Biesenthal, 2001; Pedras and Chumala, 2005; Pedras et al., 1994).
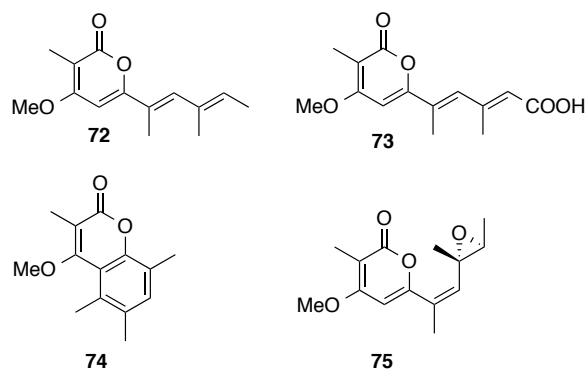
Figure 1.8   Structures of phomapyrones A (**72**), F (**74**), G (**75**) and infectopyrone (**73**).

The structures of phomapyrone A (**72**) and infectopyrone (**73**) were confirmed by synthesis of both compounds (**Scheme 1.13**, **Scheme 1.14**) (Geiseler and Podlech, 2012). Phomapyrone A (**72**) was synthesized by condensation of vermopyrone (**78**) with phosphonium salt **77** in present of BuLi (**Scheme 1.13**) (Geiseler and Podlech, 2012). None of the phomapyrones or infectopyrone (**73**) showed phytotoxic activity (Pedras et al., 2009a).
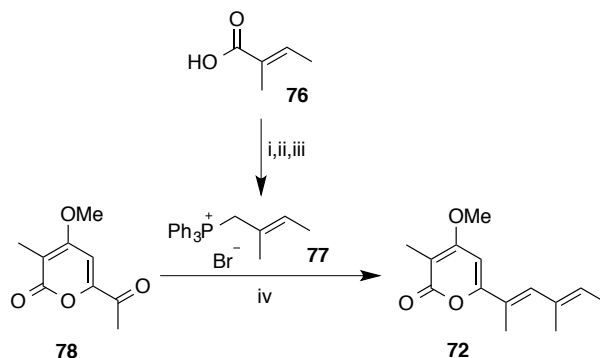
Scheme 1.13 Synthesis of phomapyrone A (**72**). Reagents and conditions: (i) LiAlH$_4$, Et$_2$O, 0 °C, 77%; (ii) PBr$_3$, Et$_2$O, 0 °C, 51%; (iii) Ph$_3$P, CH$_3$CN, 120 °C, 75%; (iv) BuLi, THF, 0 °C, 78% (Geiseler and Podlech, 2012).

Infectopyrone (**73**) was obtained from phomapyrone D (**79**) in 53% overall yield (Geiseler and Podlech, 2012). The reaction was initiated by condensation of phomapyrone D

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(**79**) with tert-butyl diethyl phosphonoacetate in the presence of BuLi (Geiseler and Podlech, 2012).
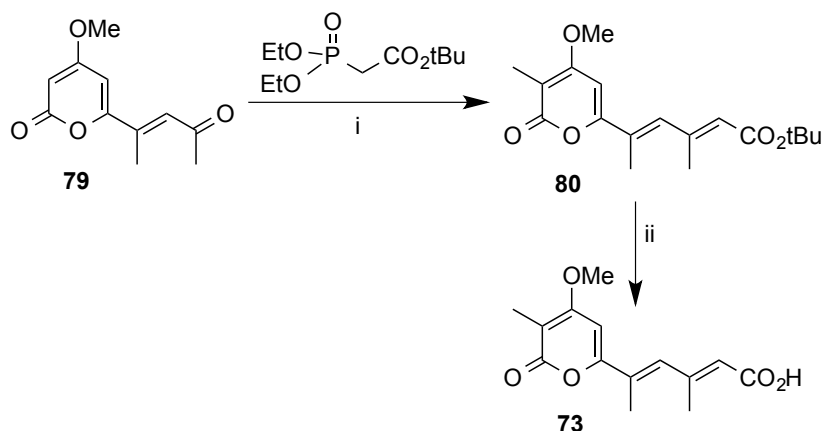
Scheme 1.14 Synthesis of infectopyrone (**73**). Reagents and conditions: (i) BuLi, THF, 0 °C, 78%; (ii) TFA, 98% (Geiseler and Podlech, 2012).

Siderophores are low molecular mass Fe^{3+} chelators (Haas et al., 2008). In fungi, one of the mechanisms of iron uptake is mediated by siderophores (Haas et al., 2008). All fungal siderophores reported so far are hydroxamates (Haas et al., 2008). *A. brassicicola* produces siderophores (Haas et al., 2008) that are responsible for iron storage. Siderophores are important in the virulence of *A. brassicicola*. A mutant of *A. brassicicola* deficient in siderophores production (Δ*Abnps6*) was less virulent on *A. thaliana* than the wild type isolate (Oide et al., 2006). An example of siderophore produced by *A. brassicicola* is shown in **Figure 1.9**.
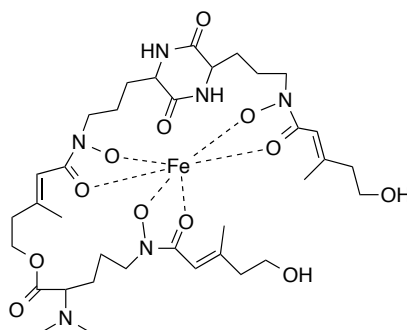
Figure 1.9   Example of a siderophore of *Alternaria brassicicola*.

1.2.2.2   Mutants of Alternaria brassicicola

Camalexin (**1**) is one of the phytoalexins produced by several wild crucifer species (Pedras et al., 2011b). Camalexin (**1**) showed strong toxicity to *A. brassicicola* (Pedras and Abdoli, 2013). Probably the toxicity of camalexin (**1**) to *A. brassicicola* is due to cell membrane damage (Joubert et al., 2011). Mutants of *A. brassicicola* (*AbSlt2*Δ) deficient in production of a kinase (Slt2) were hypersensitive to camalexin (**1**) and brassinin (**9**) (Joubert et al., 2011).

Brassinin (**9**) was detoxified to indole-3-methanamine (**28**) and *N'*-acetylindole-3-methanamine (**81**) by *A. brassicicola* (**Scheme 1.15**) (Pedras et al., 2011b). The role of brassinin (**9**) detoxification in the virulence of *A. brassicicola* to *Brassica* species was determined by disruption of the genes that encode a brassinin detoxifying enzyme (Srivastava et al., 2013). Mutants of *A. brassicicola* unable to detoxify brassinin (**9**) (Δ*bdtf1)* were less virulent on *Brassica* species than wild type isolates (Srivastava et al., 2013). Mutants caused leaf lesions about 70% smaller than the wild type on *Brassica juncea, Brassica oleracea* var. *botrytis* and *Brassica rapa* var. *pekinensis*. The virulence of these mutants on *A. thaliana*, which does not produce brassinin (**9**), was comparable to the wild type isolate of *A. brassicicola* (Srivastava et al., 2013). In general, the mutants of *A. brassicicola* that are not successful to metabolize brassinin (**9**) were less virulent in *Brassica juncea, Brassica oleracea* var. *botrytis* and *Brassica rapa* var. *pekinensis*. (Srivastava et al., 2013).

Scheme 1.15 Detoxification of the phytoalexin brassinin (**9**) by *Alternaria brassicicola* (Pedras et al., 2009a).

1.3 Metabolism of phytoalexins by fungal pathogens

In the interaction of plants with their environment, they use secondary metabolites to respond to stress. As mentioned in **Section 1.2.1.1** phytoalexins are an important group of secondary metabolites with multiple roles in protecting plants (Pedras et al., 2011b). For example, it was recently demonstrated that some cruciferous phytoalexins inhibited detoxification of cruciferous phytoalexins by inhibiting the corresponding detoxifying enzymes (Pedras and Minic, 2014; Pedras et al., 2010a). Susceptibility of plants to certain pathogens is partly related to metabolism and detoxification of phytoalexins by pathogen (Pedras et al., 2011b; Pedras and Ahiahonu, 2005). Metabolism of cruciferous phytoalexins by plant pathogens was reviewed by Pedras and co-workers (Pedras et al., 2011b), but here the metabolism of cruciferous phytoalexins of immediate interest to my PhD work is reviewed.

1.3.1 Phytoalexins from cruciferous plants

Fungal plant pathogens were reported to detoxify cruciferous phytoalexins (Pedras et al., 2011b). It was shown that the virulence of some pathogens is correlated to the ability of pathogen to detoxify phytoalexins (Srivastava et al., 2013). The biotransformations of cruciferous phytoalexins carried out by important cruciferous pathogens such as *L. maculans*, *L. biglobosa*, *R. solani*, *S. sclerotiorum*, *A. brassicicola*, and *B. cinerea* were investigated (Pedras et al., 2011b).

Camalexin (**1**) was found to be metabolized by *R. solani* (Pedras and Khan, 1997), *S. sclerotiorum* (Pedras and Ahiahonu, 2002), *B. cinerea* (Pedras et al., 2011a) and *A. brassicicola* (Pedras and Abdoli, 2013) as summarized in **Scheme 1.16**. *L. maculans* and *A. brassicae* were not able to metabolize camalexin (**1**) (Pedras et al., 1998). The detoxification pathway of camalexin (**1**) in *A. brassicicola* (Pedras and Abdoli, 2013) was identical to that used by *B. cinerea* (Pedras et al., 2011a). Camalexin (**1**) was transformed by both species to indole-3-thiocarboxamide (**82**), which was metabolized further to indole-3-carboxylic acid (**84**) *via* indole-3-carbonitrile (**83**). However, the rate of transformation of camalexin (**1**) in cultures of *A.*

brassicicola was much slower than that observed in *B. cinerea* (Pedras et al., 2011a; Pedras and Abdoli, 2013). Camalexin (**1**) was detoxified by *S. sclerotiorum* to 6-oxy-(*O-β-*glucopyranosyl)camalexin (**86**) *via* 6-hydroxycamalexin (**85**) (Pedras and Ahiahonu, 2002). Camalexin (**1**) was transformed to 5-hydroxycamalexin (**87**) by *R. solani*, which was further transformed to 5-hydroxy-2-formamidophenyl-2'-thiazolylketone (**88**) and 5-hydroxyindole-3-carbonitrile (**89**) (**Scheme 1.16**) (Pedras and Khan, 1997).
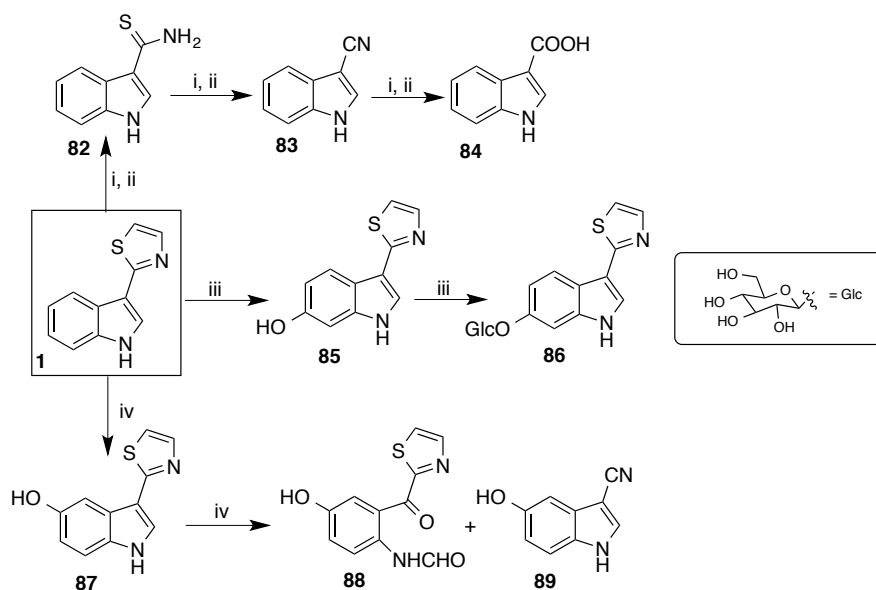
Scheme 1.16 Detoxification of the phytoalexin camalexin (**1**) by plant pathogens: (i) *Botrytis cinerea* (Pedras et al., 2011a); (ii) *Alternaria brassicicola* (Pedras and Abdoli, 2013); (iii) *Sclerotinia sclerotiorum* (Pedras and Ahiahonu, 2002) (iv) *Rhizoctonia solani* (Pedras and Khan, 1997).

1-Methylcamalexin (**2**) was detoxified by *R. solani* to 1-methylindole-3-carbonitrile (**92**), 1-methylindole-3-carboxamide (**97**) and 2-(1-methyl-3-indolyl)-oxazoline (**96**) (**Scheme 1.17**) (Pedras and J. Liu, 2004). Recently, metabolism of 1-methylcamalexin (**2**) was investigated in *A. brassicicola* (Pedras and Abdoli, 2013). *A. brassicicola* carried out the oxidative degradation of the thiazole ring to the corresponding thiocarboxamide **90** that was further metabolized to nitrile **92** and acid **94** (**Scheme 1.17**) (Pedras and Abdoli, 2013).
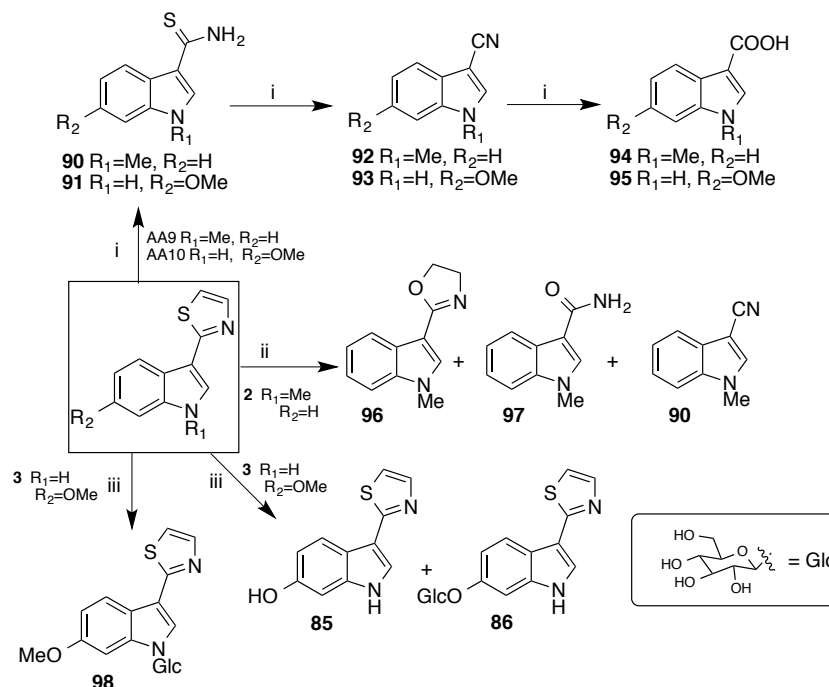
Scheme 1.17 Detoxification of the phytoalexins 1-methylcamalexin (**2**) and 6-methoxycamalexin (**3**) by plant pathogens: (i) *Alternaria brassicicola* (Pedras and Abdoli, 2013); (ii) *Rhizoctonia solani* (Pedras and Liu, 2004); (iii) *Sclerotinia sclerotiorum* (Pedras and Ahiahonu, 2002).

6-Methoxycamalexin (**3**) was metabolized by *S. sclerotiorum* through two different pathways (Pedras and Ahiahonu, 2002) (**Scheme 1.17**). In one pathway, 6-methoxycamalexin (**3**) was metabolized through demethylation of the methoxy group to 6-hydroxycamalexin (**85**) followed by glucosylation of the hydroxyl group to compound **86**. 6-Methoxycamalexin (**3**) was also metabolized by *S. sclerotiorum* to the *N*-glucosylated product **98**, a minor metabolite (Pedras and Ahiahonu, 2002). Metabolism of 6-methoxycamalexin (**3**) in *A. brassicicola* yielded thiocarboxamide **91** as a result of oxidative degradation of its thiazole ring (Pedras and Abdoli, 2013). Thiocarboxamide **91**was further metabolized by *A. brassicicola* to the corresponding nitrile **93** and acid **95** (**Scheme 1.17**) (Pedras and Abdoli, 2013).

The metabolism of brassilexin (**7**) by two fungal species was investigated (**Scheme 1.18**). Brassilexin (**7**) was metabolized by *L. maculans* (virulent on canola) to enamine **99** through reduction of N-S bond of its isothiazole ring, then enamine **99** was metabolized further to sulfonic acid **100** (Pedras and Suchy, 2005). Brassilexin (**7**) was detoxified to the *N*-glucosylated

product **101** by *S. sclerotiorum* utilising a glucosyl transferase (**Scheme 1.18**) (Pedras and Hossain, 2006).
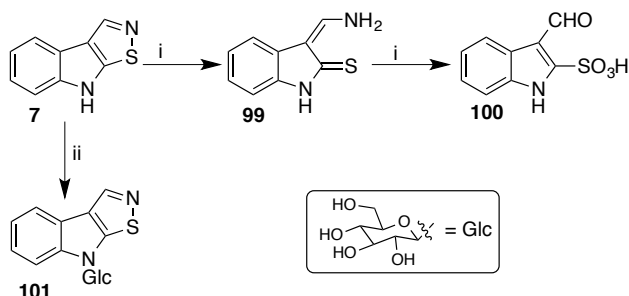
Scheme 1.18 Detoxification of the phytoalexin brassilexin (**7**) by plant pathogens: (i) *Leptosphaeria maculans* (Pedras and Suchy, 2005); (ii) *Sclerotinia sclerotiorum* (Pedras and Hossain, 2006).

The transformations of cyclobrassinin (**4**) by *L. maculans* (Pedras, 1998), *L. biglobosa* (Pedras and Okanga, 1999), *R. solani* (Pedras and Okanga, 1999) and *S. sclerotiorum* (Pedras et al., 2004a) were investigated (**Scheme 1.19**). Cyclobrassinin (**4**) was metabolized to the phytoalexin dioxibrassinin (**103**) by *L. maculans* (Pedras, 1998) and brassilexin (**7**) (Pedras and Okanga, 1999) by *L. biglobosa*. The metabolism of cyclobrassinin (**4**) by *S. sclerotiorum* was *via* glycosylation to the *N*-glucosylated product **102** (Pedras et al., 2004a). *R. solani* transformed cyclobrassinin (**4**) to the phytoalexin brassicanal A (**6**), which was oxidized further to compound **105** *via* brassicanal A (**6**) (Pedras and Okanga, 1999).
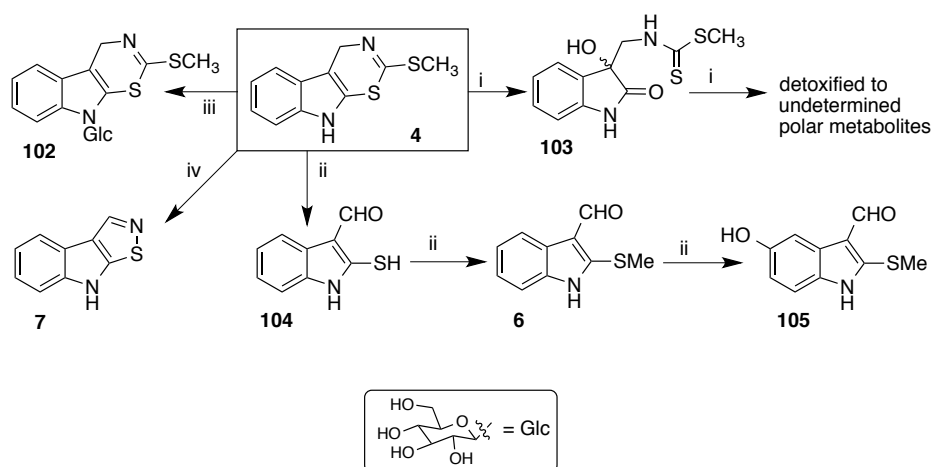
Scheme 1.19 Detoxification of the phytoalexin cyclobrassinin (**4**) by plant pathogens: (i) *Leptosphaeria maculans* (Pedras, 1998); (ii) *Rhizoctonia solani* (Pedras and Okanga, 1999); (iii) *Sclerotinia sclerotiorum* (Pedras et al., 2004a); (iv) *Leptosphaeria biglobosa* (Pedras, 2014; Pedras and Okanga, 1999).

The metabolism of the phytoalexin brassinin (**9**) by several cruciferous fungi such as *L. maculans*, *A. brassicicola*, *L. biglobosa*, *B. cinerea* and *S. sclerotiorum* were investigated as summarized in **Scheme 1.20** (Pedras et al., 2011b). It was indicated that *A. brassicicola*, *L. maculans* (virulence on mustard), *L. biglobosa* and *B. cinerea* transformed brassinin (**9**) to identical products. These pathogens metabolized brassinin (**9**) to indole-3-methanamine (**28**) followed by N_b-acetyl-indole-3-methanamine (**81**) (Pedras et al., 2011a; 2009a; 2007a). Brassinin (**9**) was transformed by *S. sclerotiorum* to the *N*-glucosylated compound **106** (Pedras et al., 2004a). Brassinin (**9**) was oxidized to indole-3-carboxaldehyde (**26**) by *L. maculans* (virulent on canola) followed by further oxidation to indole-3-carboxylic acid (**84**) (**Scheme 1.20**) (Pedras and Jha, 2006).
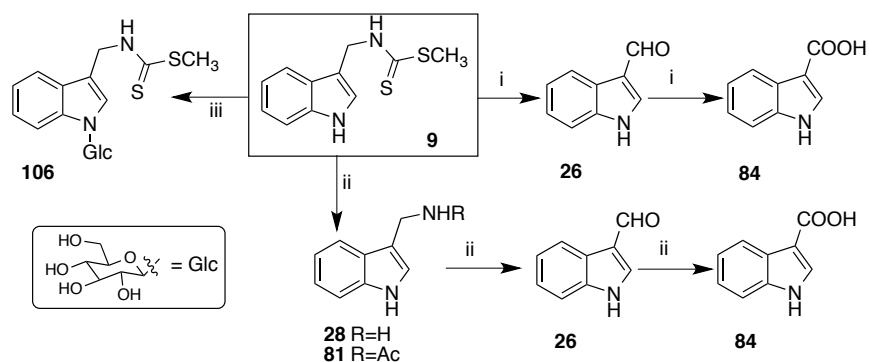
Scheme 1.20 Detoxification of the phytoalexin brassinin (**9**) by plant pathogens: (i) *Leptosphaeria maculans* (virulence on canola); (ii) *Alternaria brassicicola*, *L. biglobosa* and *Botrytis cinerea* (Pedras et al., 2011a; 2009a; 2007a); (iii) *Sclerotinia sclerotiorum* (Pedras et al., 2004a).

1.3.2 Phytoalexins from non-cruciferous plants

To date, several examples reported in the literature demonstrate that fungal pathogens can efficiently detoxify phytoalexins from other plant families (Pedras and Ahiahonu, 2005). Metabolism and detoxification of phytoalexins from non-cruciferous plants was comprehensively reviewed in 2005 (Pedras and Ahiahonu, 2005) and here only the work reported after 2005 is reviewed.

The phytoalexin daidzein (**107**), isolated from Colombian bean (*Phaseolus vulgaris*) (Durango et al., 2002) was metabolized by the fungus *Aspergillus oryzae* to the potent antioxidant 8-hydroxydaidzein (**108**) (**Scheme 1.21**) (Seo et al., 2013). Metabolism of daidzein (**107**) to 8-hydroxydaidzein (**108**) in *A. oryzae* was identical to *Aspergillus saitoi* (**Scheme 1.21**) (Esaki et al., 1998) (Pedras and Ahiahonu, 2005).
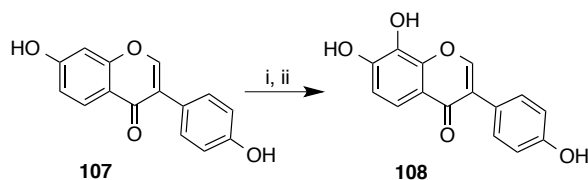
Scheme 1.21 Detoxification of daidzein (**107**) by (i) *Aspergillus oryzae* (Seo et al., 2013); (ii) *Aspergillus saitoi* (Esaki et al., 1998; Pedras and Ahiahonu, 2005).

Sakuranetin (**109**) is a phytoalexin isolated from rice plants (*Oryza sativa*) infected by *Pyricularia oryzae* (Dillon et al., 1997). The metabolism of the phytoalexin sakuranetin (**109**) by *Magnaporthe oryzae* (rice blast fungus) indicated that the phytoalexin was transformed, although no metabolites were isolated from the cultures (Hasegawa et al., 2010). Later on, analysis of extracts of cultures of *M. oryzae* incubated with sakuranetin (**109**) showed that it was metabolized to naringenin (**110**) (**Scheme 1.22**) (Hasegawa et al., 2014).
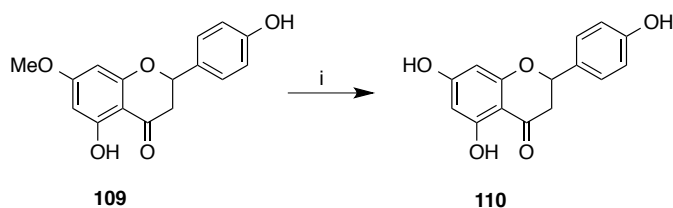
Scheme 1.22 Detoxification of sakuranetin (**109**) by (i) *Magnaporthe oryzae* (Hasegawa et al., 2014).

The phytoalexin genistein (4′,5,7-trihydroxyisoflavone) (**111**) was metabolized by *Armillaria mellea*, causal agent of root rot, to five different compounds; 4-hydroxyphenylacetic acid (**112**), 2,5-dihydroxyphenylacetic acid (homogentisic acid) (**113**), its lactone 5-hydroxy-2(3H)-benzofuranone (**114**), 1,4-benzoquinone (**115**) and 1,3,5-trihydroxybenzene (**116**) (Curir et al., 2006) (**Scheme 1.23**). All metabolites caused lower growth inhibition against mycelial growth of *A. mellea* than the parent compound. The pathway of detoxification of genistein (**111**) was proposed as shown in **Scheme 1.23** (Curir et al., 2006).
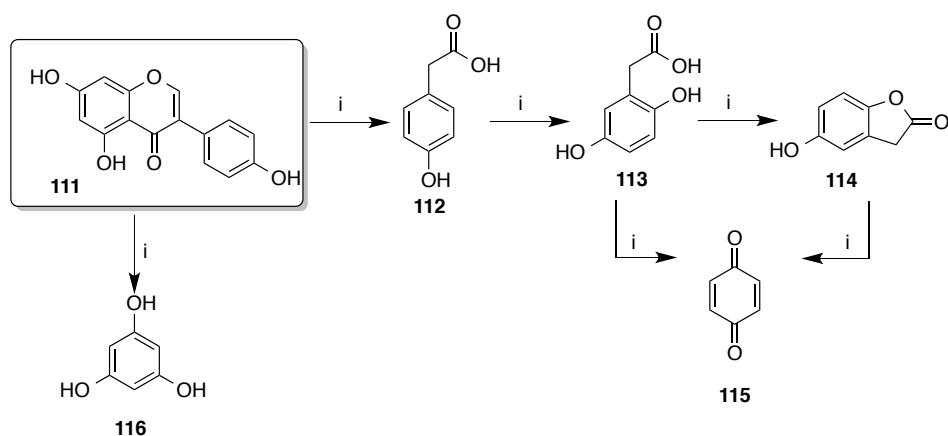
Scheme 1.23 Detoxification of genistein (**111**) by (i) *Armillaria mellea* (Curir et al., 2006).

The metabolism of momilactone A (**117**), the major phytoalexin from rice, was investigated in 2010: although no metabolites were isolated from the cultures incubated with momilactone A (**117**) (Hasegawa et al., 2010). Later on, it was reported that momilactone A (**117**) was transformed to 3,6-dioxo-19-nor-9β-pimara-7,15-diene (**118**) by *M. oryzae* (**Scheme 1.24**) (IMAI et al., 2012). Compound **118** was further metabolized to undetermined metabolite(s) by *M. oryzae.* The antifungal activity of compound **118** was the same as that of momilacton A (**117**). 3,6-Dioxo-19-nor-9β-pimara-7,15-diene (**118**) was proposed as a precursor for the detoxified metabolites.
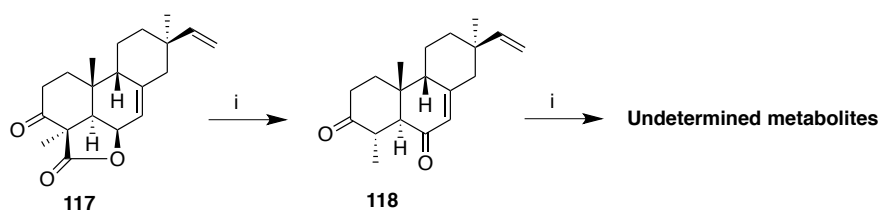
Scheme 1.24 Detoxification of momilacton A (**117**) by (i) *Magnaporthe oryzae* (Imai et al., 2012).

The metabolism of resveratrol (**119**) by *Beauveria bassiana* and *Penicillium chrysogenium* was investigated (Herath et al., 2013). Resveratrol (**119**) was metabolized by *B.*

bassiana to 5-methoxyresvertarol-3-O-glucoside (**120**) and 5-hydroxyresveratrol-3-sulphate (**122**) (Herath et al., 2013). *P. chrysogenium* metabolized resveratrol (**119**) to 5-methoxyresveratrol-3-sulphate (**121**) and 5-hydroxyresveratrol-3-sulphate (**122**) (Herath et al., 2013).
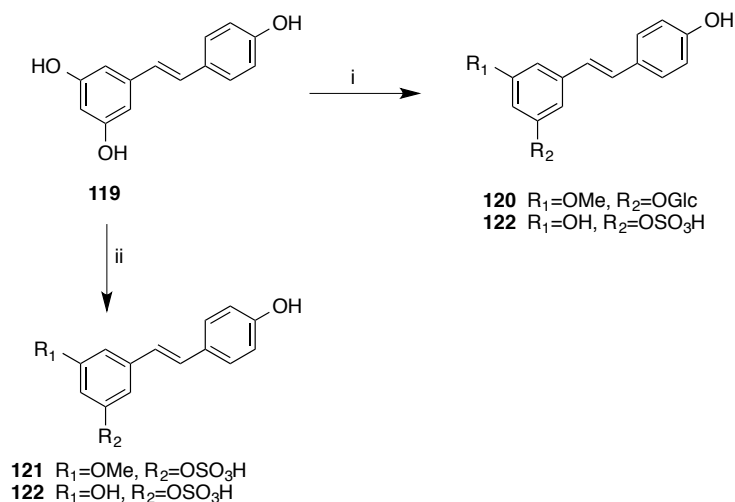
Scheme 1.25 Detoxification of resveratrol (**119**) by (i) *Beauveria bassiana*; (ii) *Penicillium chrysogenium* (Herath et al., 2013).

Stilbenes are a group of antifungal phenolic compounds have been found in spruce and other species of the family Pinaceae (Underwood and Pearce, 1992). The stilbene-type phytoalexin astringin (**123**), biosynthesized from resveratrol (**119**) (Jeandet et al., 2014) (Hammerbacher et al., 2011), is transformed by two different isolates of *Ceratocystis polonica* (**Scheme 1.26**) (Hammerbacher et al., 2013). Metabolism of astringin (**123**) yielded compounds **128**, **124**, **125**, and **129** (Hammerbacher et al., 2013). Metabolites **124**, **125**, **128** and **129** were transformed further by *C. polonica* to piceatannol lactone **130** and piceatannol dimers **132** and **133** (**Scheme 1.26**).
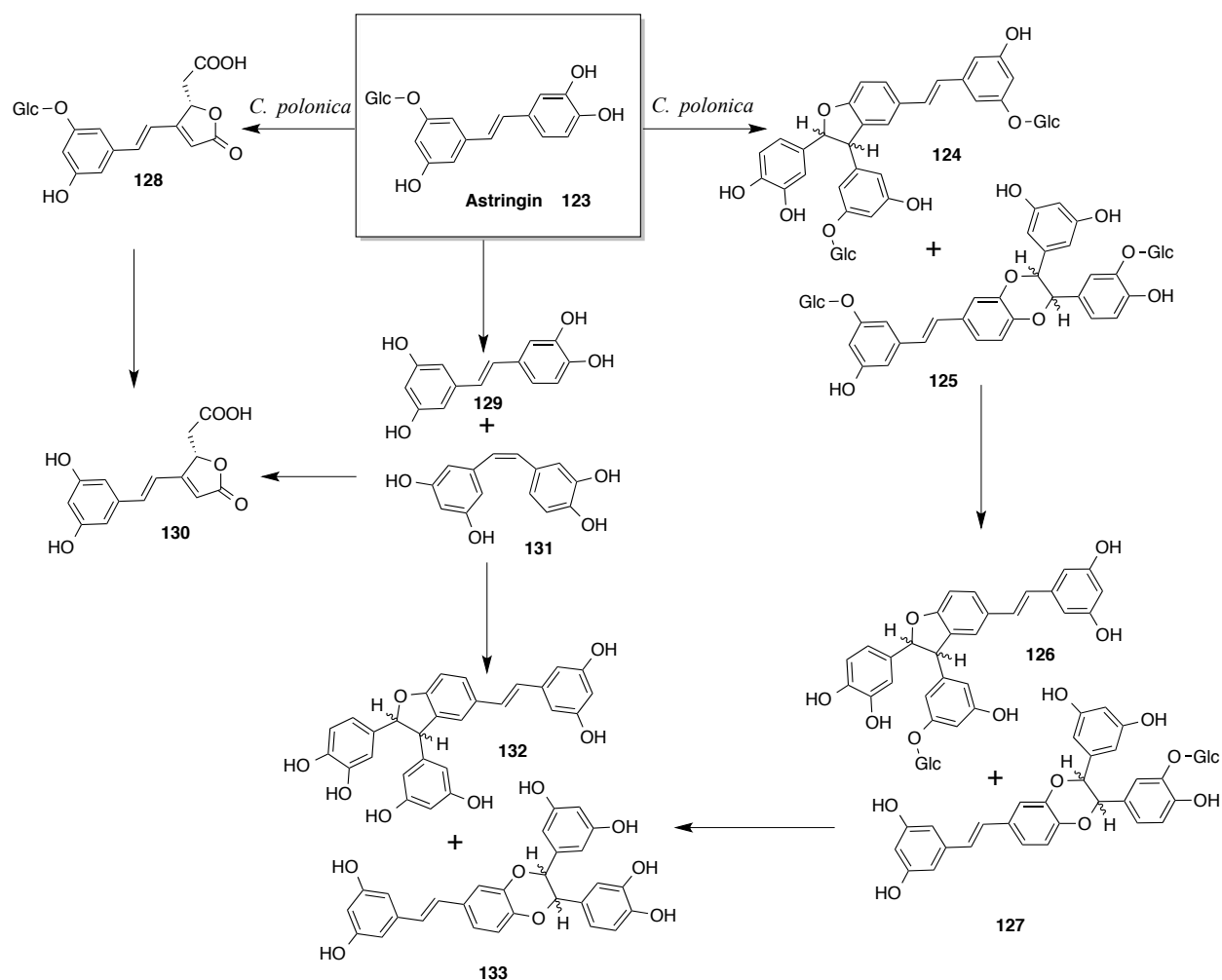
Scheme 1.26 Detoxification of astringin (**123**) by *Ceratocystis polonica* (Hammerbacher et al., 2013).

The metabolism of glyceollin (**134**), a soybean phytoalexin, was investigated in different plant pathogens (Lygin et al., 2010). Metabolism of glyceollin (**134**) in *Cercospora sojina*, *Diaporthe phaseolorum* var. *meridionales*, *Macrophomina phaseolina*, *Phialophora gregata* and *R. solani* yielded compound **135** (**Scheme 1.27**) (Lygin et al., 2010). Glyceollin (**134**) was metabolized to compound **136** in cultures of *Sclerotinia sclerotiorum* (Lygin et al., 2010).
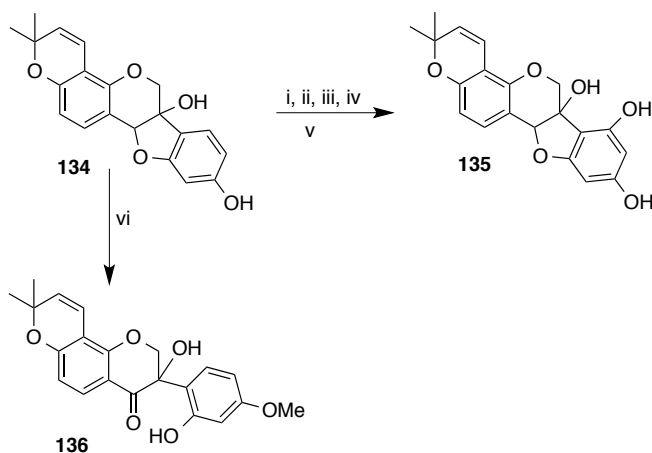
Scheme 1.27 Detoxification of glyceollin (**134**) by: (i) *Cercospora sojina*; (ii) *Diaporthe phaseolorum* var. *meridionales*; (iii) *Macrophomina phaseolina*; (iv) *Phialophora gregata*; (v) *Rhizoctonia solani*; (vi) *Sclerotinia sclerotiorum* (Lygin et al., 2010).

1.4 Inhibition of detoxification of cruciferous phytoalexins

Plants resist pathogens using both constitutive and induced defences. Phytoalexins, an induced defence, have been studied for many years; however, the specific roles of these induced chemicals are not fully understood. Recently, it has been shown that cruciferous phytoalexins can inhibit the detoxification of other phytoalexins by cruciferous pathogens. Detoxification of cruciferous phytoalexins by important cruciferous pathogens has been studied (Pedras et al., 2011b) and is an on-going investigation. Preventing these detoxification reactions using inhibitors of enzymes responsible for these detoxifications is also under investigation. PALDOXINS (phytoalexin detoxification inhibitors) are a new generation of synthetic compounds that can selectively inhibit phytoalexin detoxifying enzymes, with minimal effect on other organisms and environment (Pedras, 2014; Pedras et al., 2011b). Some of the enzymes involved in selective detoxification of cruciferous phytoalexins were recently reported (Pedras, 2014).

1.4.1   11Detoxifying enzymes

Five phytoalexin detoxifying enzymes were isolated and characterized from four fungal species (Pedras and Minic, 2014; Pedras et al., 2008a; 2009c; Sexton et al., 2009). Four of these enzymes are responsible for detoxification of brassinin (**9**) (Pedras et al., 2009c; 2008a; Sexton et al., 2009) and one is responsible for detoxification of cyclobrassinin (**4**) (Pedras and Minic, 2014). Pioneering work led to the isolation of brassinin oxidase from *L. maculans* (BOLm) (Pedras et al., 2008a). BOLm responsible for oxidative detoxification of brassinin (**9**) to indole-3-carboxaldehyde (**26**) (**Scheme 1.28**) was the first phytoalexin detoxifying enzyme ever characterized. Production of BOLm in fungal cultures required induction with specific compounds such as 3-phenylindole (**234**) or camalexin (**1**) (Pedras et al., 2008a; 2005). Protein extracts of control cultures of *L. maculans* showed very low BO activity (Pedras et al., 2008a). BOLm was purified from mycelia of *L. maculans* using brassinin (**9**) as substrate (Pedras et al., 2008a).
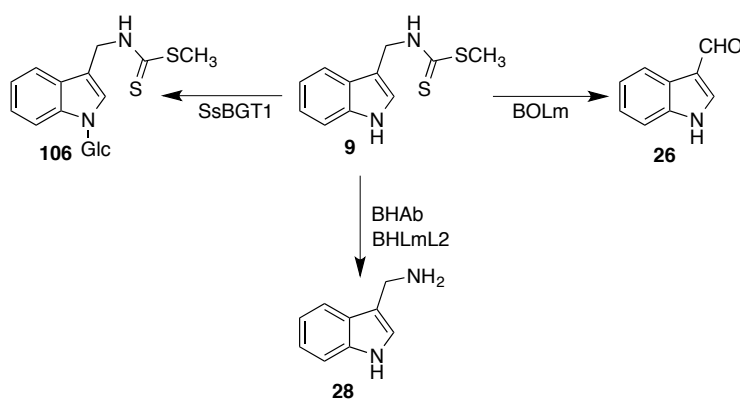
Scheme 1.28 Transformation of brassinin (**9**) by brassinin oxidase from *Leptosphaeria maculans* (isolate virulent on canola) (BOLm); Brassinin hydrolase from *Alternaria brassicicola* (BHAb); brassinin hydrolase from *Leptosphaeria maculans* (virulent on mustard) (BHLmL2) and brassinin glucosyl transferase from *Sclerotinia sclerotiorum* (SsBGT1).

Two brassinin hydrolases were isolated from *L. maculans* (isolate L2, virulent on mustard) (BHLmL2) and *A. brassicicola* (BHAb) (Pedras et al., 2009c). BHAb is a dimeric protein with the molecular mass of 120 kDa, while BHLmL2 is a tetrameric protein with mass of

220 kDa (Pedras et al., 2009c). BHLmL2 and BHAb catalyzed the detoxification of brassinin (**9**) to indole-3-methaneamine (**28**) (**Scheme 1.28**). Similar to BOLm, brassinin hydrolases are non-constitutive enzymes induced by camalexin (**1**) and 3-phenylindole (**234**). BHs showed a high degree of substrate specificity (Pedras et al., 2009c).

Brassinin glucosyl transferase (BGT1) from *Sclerotinia sclerotiorum* (SsBGT1) is inducible and involved the detoxification of brassinin (**9**) via glucosylation of the nitrogen of indole (**Scheme 1.28**). The enzyme was expressed in *Saccharomyces cerevisiae* and purified (Sexton et al., 2009). Three substrates were found for SsBGT1, the phytoalexins cyclobrassinin (**4**) and 6-methoxycamalexin (**3**), and 3-phenylindole (**234**) (Sexton et al., 2009).

Recently cyclobrassinin hydrolase was isolated from *A. brassicicola* (CHAb) (Pedras and Minic, 2014). The enzyme catalyzed transformation of cyclobrassinin (**4**) to *S*-methyl [(2-sulfanyl-1H-indolyl-3)methyl]carbamothioate (**137**) (**Scheme 1.29**).
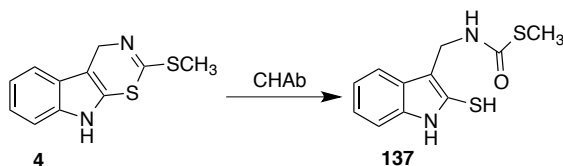
Scheme 1.29 Transformation of cyclobrassinin (**4**) by cyclobrassinin hydrolase from *Alternaria brassicicola* (CHAb).

CHAb is an inducible enzyme and its production in fungal cultures required induction with camalexin (**1**) (Pedras and Minic, 2014). Protein extracts of the control cultures of *A. brassicicola* showed very low CHAb activity (Pedras and Minic, 2014). Cyclobrassinin hydrolase is a tetrameric protein with a molecular mass of 330 kDa (Pedras and Minic, 2014).

1.4.2 Inhibitors

1.4.2.1 *Phytoalexins*

In addition to their antifungal activities, phytoalexins can inhibit phytoalexin detoxifying enzymes produced by pathogens (Pedras, 2014). To identify potential inhibitors of phytoalexin detoxifying enzymes, cruciferous phytoalexins were screened using purified enzymes involved in brassinin detoxification. Camalexin (**1**), 6-methoxycamalexin (**3**), cyclobrassinin (**4**), brassilexin (**7**), spirobrassinin (**11**) and wasalexins (**138** and **139**) inhibited BOLm activity (Pedras et al., 2010a; 2009b; 2008a). The phytoalexins brassitin (**141**) and cyclobrassinin (**4**) inhibited BHs activity (Pedras et al., 2012), whereas the phytoalexins camalexin (**1**), brassicanal A (**6**), brassilexin (**7**), dioxibrassinin (**103**) and sinalexin (**140**) inhibited CHAb (Pedras and Minic, 2014). The inhibitors of phytoalexin detoxifying enzymes identified to date are summarized in **Table 1.1**.
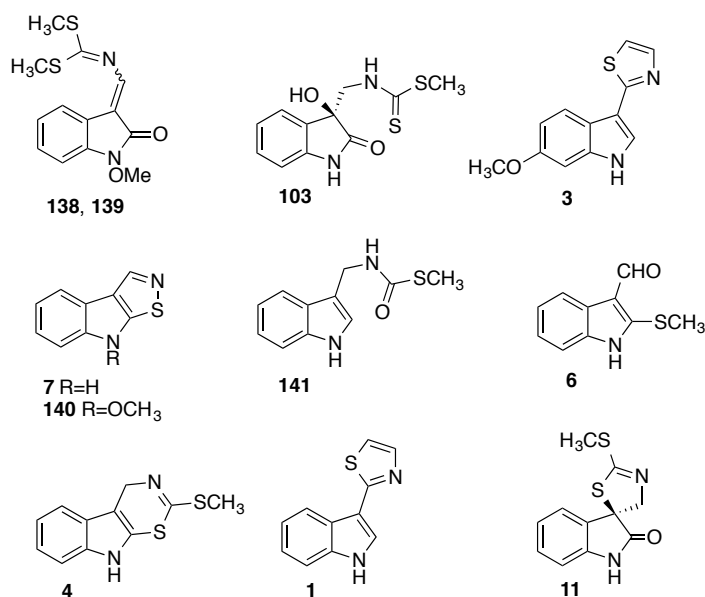
Figure 1.10   Structures of phytoalexin that are inhibitors of phytoalexin detoxifying enzymes.

Table 1.1 Effect of phytoalexins on inhibition of phytoalexin detoxifying enzymes

Compound (#)	Inhibition (%)				
	Conc. (mM)	BOLm	BHLmL2	BHAb	CHAb
Camalexin (**1**)	0.30	53 ± 4	n. d.	n. d.	65 ± 5
	0.10	30 ± 4			52 ± 6
6-Methoxycamalexi (**3**)	0.30	63 ± 5	n. d.	n. d.	n. d.
	0.10	41 ± 6			
1-Methylcamalexin (**2**)	0.30	n. i.	n. d.	n. d.	n. d.
	0.10	n. i.			
Cyclobrassinin (**4**)	0.30	37 ± 8	n. d.	26 ± 4	n. d.
	0.10	23 ± 6		16 ± 2	
Brassilexin (**7**)	0.30	16 ± 2	n. i.	n. i.	93 ± 2
	0.10	8 ± 2	n. i.	n. i.	85 ± 2
Wasalexin A & B (**138** & **139**)	0.30	14 ± 4	n. d.	n. d.	n. d.
	0.10	8 ± 5			
Rutalexin (**5**)	0.30	n. d.	n. i.	n. i.	n. d.
	0.10	n. i.	n. i.	n. i.	
Erucalexin (**13**)	0.30	n. d.	n. i.	n. i.	n. d.
	0.10		n. i.	n. i.	
Brassicanal A (**6**)	0.30	n. d.	n. i.	n. i.	43 ± 4
	0.10		n. i.	n. i.	20 ± 3
Brassinin (**9**)	0.30	n. d.	n. d.	n. d.	n. i.
	0.10				n. i.
Sinalexin (**140**)	0.30	n. d.	n. d.	n. d.	52 ± 5
	0.10				30 ± 2
Spirobrassinin (**11**)	0.30	n. d.	n. d.	n. d.	n. i.
	0.10				n. i.
Dioxibrassinin (**103**)	0.30	n. d.	n. d.	n. d.	58 ± 5
	0.10				40 ± 5

n. i. = no inhibition
n. d. = not determined

1.4.2.2 Synthetic inhibitors

Evaluation of synthetic compounds uncovered some inhibitors of phytoalexins detoxifying enzymes. The chemical structures of the tested compounds were designed based on the structures of phytoalexins that displayed inhibitory activity.

Brassinin oxidase from Leptosphaeria maculans (BOLm)

A library of compounds was synthesized based on the structure of camalexin (**1**, 53% at 0.30 mM). The inhibitory activity of these compounds was determined using purified BOLm (Pedras et al., 2009b). Results of the enzymatic assays indicated that 5-methoxycamalexin (**145**, 72% at 0.30 mM) was the strongest inhibitor followed by 5-fluorocamalexin (**146**, 63% at 0.30 mM). Amongst the tested derivatives of camalexin (**1**), 6-fluorocamalexin (**147**, 46% at 0.30 mM) was the weakest inhibitor of BOLm. In general, 5-substituted camalexins inhibited BOLm to a larger extend than their 6-substituted derivatives (Pedras et al., 2009b). Using naphthalene instead of indole and replacing the thiazole ring of camalexin (**1**) with isothiazole afforded additional potential inhibitors of BOLm; among these, isothiazoles **142** (21% at 0.30 mM) and **143** (42% at 0.30 mM) were found to moderately inhibit BO activity (**Figure 1.11**) (Pedras et al., 2009b).
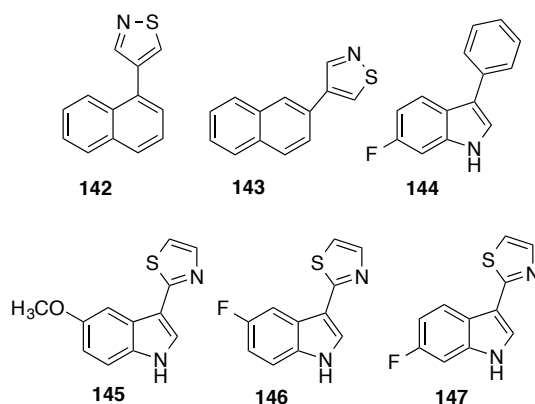
Figure 1.11   Structures of BOLm inhibitors, compounds **142–147**.

Another group of potential inhibitors of BOLm was designed based on the structures of brassilexin (**7**) and wasalexins A (**138**) and B (**139**) (Pedras et al., 2010a). Among the derivatives of wasalexins A (**138**) and B (**139**) (14% at 0.30 mM), compound **148** (18% at 0.30 mM) and **149** (14% at 0.30 mM) were shown to inhibit BOLm (**Figure 1.12**) (Pedras et al., 2010a). Among the derivatives of brassilexin (**7**), compounds **150-158** showed inhibitory activity against BOLm (**Figure 1.12**). 6-Chlorobrassilexin (**153**, 66% at 0.30 mM) and 6-bromobrassilexin (**155**, 63% at 0.30 mM) showed significant inhibitory activity (Pedras et al., 2010a). In general, 6-substituted brassilexins were found to be more inhibitory than 5-substuted brassilexins (Pedras et al., 2010a).
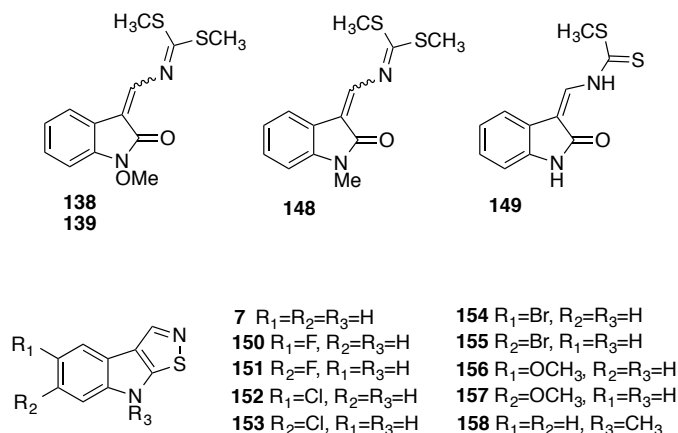
Figure 1.12 Structures of BOLm inhibitors based on scaffold of brassilexin (**7**) and wasalexins A (**138**) and B (**139**) (Pedras et al., 2010a).

Thiabendazole (**159**, 25% at 0.30 mM), a commercially available fungicide, did not show significant activity against BOLm (**159**, 25% at 0.30 mM) (Pedras et al., 2008a).
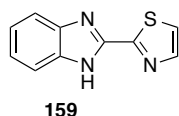
Figure 1.13 Structure of thiabendazole (**159**).

Brassinin hydrolase from Alternaria brassicicola (BHAb)

Among the synthetic compounds tested for inhibition of BHAb, methyl N-(1-naphthalenylmethyl) carbamate (**160**, 89% at 0.30 mM) was discovered as the best inhibitor (**Figure 1.14**), followed by methyl N-(1-naphthalenylmethyl) carbonate (**161**, 62% at 0.30 mM concentration) and methyl N-(2-naphthalenylmethyl) carbonate (**162**, 52% at 0.30 mM) (Pedras et al., 2012). Among the compounds synthesized based on the structure of brassinin (**9**), methyl N′-(3-indolylmethyl) carbamate (**163**, 21% at 0.30 mM) and methyl 1-methyl-N′-(3-indolylmethyl) carbamate (**164**, 46% at 0.30 mM) were identified as inhibitors of BHAb (Pedras et al., 2012). In general, compounds **160**, **161** and **162** with a naphthalene ring were more inhibitory than compounds **163** and **164** with an indolyl ring (Pedras et al., 2012).

160 X=NH, Y=O, Z=OCH$_3$
161 X=O, Y=O, Z=OCH$_3$

162

163 R=H
164 R=CH$_3$
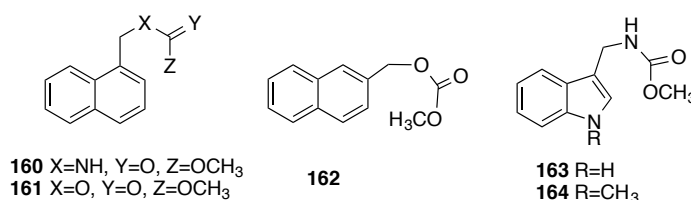
Figure 1.14   Structures of BH inhibitors (Pedras et al., 2012).

Cyclobrassinin hydrolase from Alternaria brassicicola (CHAb)

1-Methylbrassilexin (**165**) and 1-methylbrassinin (**166**) were the only synthetic compounds tested as potential inhibitors of CHAb (Pedras and Minic, 2014). 1-Methylbrassilexin (**165**, 73% at 0.30 mM) showed significant inhibition against BHAb; after the phytoalexin brassilexin (**7**, 93% at 0.30 mM) it is the strongest inhibitor of CHAb. In contrast, 1-methylbrassinin (**166**) did not show inhibitory activity against CHAb (Pedras and Minic, 2014).
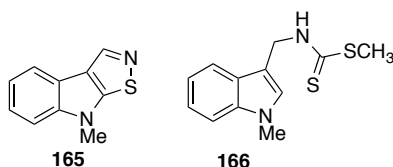
Figure 1.15   Structure of 1-methylbrassilexin (**165**) and 1-methylbrassinin (**166**).

1.5   Conclusion

The biosynthesis of phytoalexins in infected plants is an important defence mechanism of plants to protect themselves against pathogens. In turn, enzymatic detoxification of phytoalexins by pathogens is an important strategy among pathogens. As a result of detoxification reactions, plants are deprived of their natural defences, which make them more susceptible to pathogens. Inhibition of phytoalexin detoxifying enzymes could help plants accumulate sufficient amount of phytoalexins in the infected parts. Phytoalexin detoxification inhibitors (PALDOXINS) are an interesting class of synthetic compounds that could selectively inhibit enzymes responsible for metabolism of phytoalexins. Paldoxins with low antifungal and cytotoxic activity might serve as better crop protecting agents than fungicides.

This thesis describes the chemistry involved in the metabolism of cruciferous phytoalexins, their derivatives and analogues by *A. brassicicola* and the effects of camalexin related structures on brassinin oxidase (BOLm) activity.

2 RESULTS AND DISCUSSION

2.1 Transformation of phytoalexins and related structures by *Alternaria brassicicola*

Crucifers produce a wide range of secondary metabolites to protect themselves against environmental stresses such as microbial pathogens. Phytoalexins are inducible plant secondary metabolites produced during infection and usually not synthesized in healthy plants (Pedras et al., 2011b). Notwithstanding the variety of antifungal metabolites (such as phytoalexins) produced in crucifers, they are susceptible to some pathogens because of the ability of pathogens to detoxify phytoalexins (Pedras et al., 2011b). Although the transformation of cruciferous phytoalexins by plant pathogens has been investigated for more than two decades (Pedras et al., 2011b), there are many cruciferous phytoalexins whose resistance to transformation by plant pathogens is still unknown. Investigation of phytoalexin transformation by *A. brassicicola* was carried out following the general procedure summarized in **Figure 2.1** (Pedras and Abdoli, 2013).
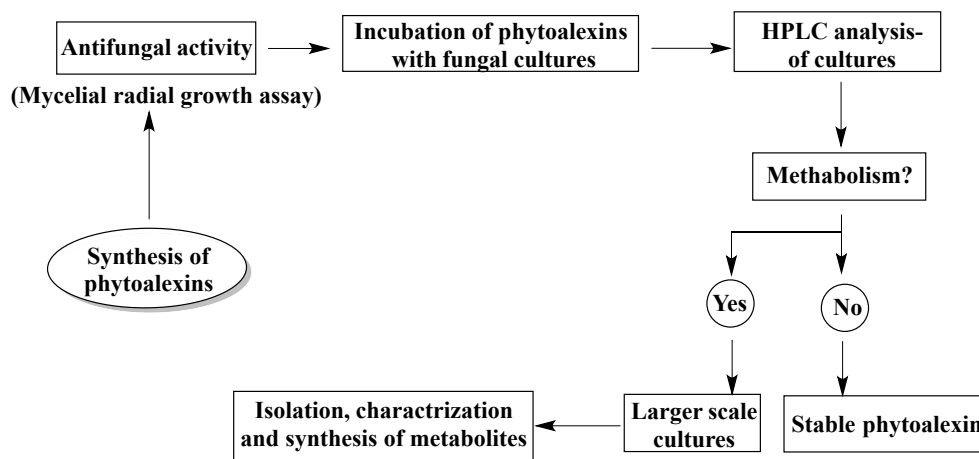
Figure 2.1   Experimental flowchart for investigation of phytoalexin transformation.

2.1.1 Camalexins and related structures

2.1.1.1 Synthesis and antifungal activity

The camalexins **1**, **2** (Pedras and Liu, 2004), **3** (Ayer et al., 1992) and related structures **167** (Ayer et al., 1992), **169** (Moody et al., 1997), **171, 172** (Pedras et al., 2009b)**,** (**Figure 2.2**) were synthesized following published procedures. Syntheses of camalexins **1**, **2** and **3** were carried out as described in **Section 3.3**.
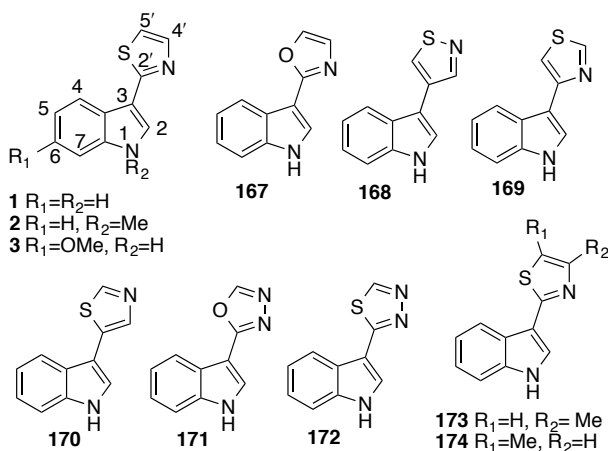
Figure 2.2   Structures of camalexins **1-3** and related structures **167-174**.

Camalexin analogues **173**, **174** and structural isomers **168** and **170** are new compounds whose syntheses have not been reported previously. Analogues **173** and **174** were synthesized as summarized in **Scheme 2.1** (Pedras and Abdoli, 2013). Compound **173** was obtained from indole-3-thiocarboxamide (**82**), which was prepared from the nitrile **83** as described in **Section 3.4.1.3.1**, upon reaction with chloroacetone in ethanol (Moody et al., 1997) under reflux, in quantitative yield. Similarly, condensation of 2-bromopropanal (**176**) with indole-3-thiocarboxamide (**82**) yielded compound **174** in 90% yield (**Scheme 2.1**) (Pedras and Abdoli, 2013). 2-Bromopropanal (**176**) was prepared by regioselective α-bromination of propionaldehyde (**175**) in 1,4-dioxane at 0 °C (**Scheme 2.1**) (Gangjee et al., 2005).
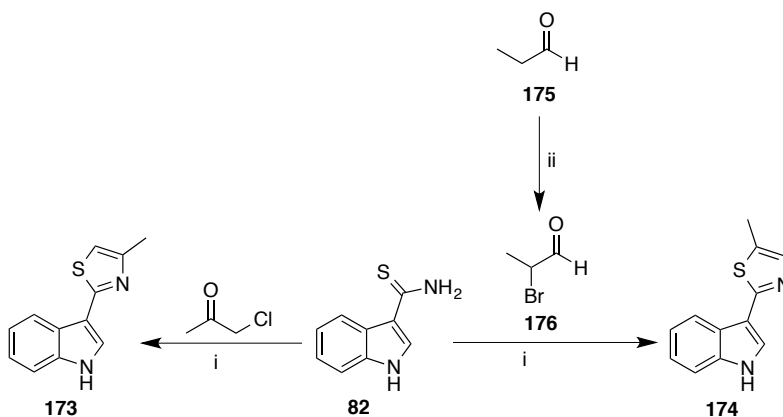
Scheme 2.1 Syntheses of 2-(1*H*-indol-3-yl)-4-methylthiazole (**173**) and 2-(1*H*-indol-3-yl)-5-methylthiazole (**174**). Solvent and conditions: (i) 95% EtOH, 80 °C, 1.5 h (**173**, 90%) and 2 h (**174**, quantitative); (ii) Br$_2$, 1,4-dioxane, 0 °C, 1 h, 62% (Gangjee et al., 2005).

Synthesis of **170** was started from 2-chloro-1-(3-indolyl)ethanone (**177**), prepared from reaction of indole (**231**) with chloroacetyl chloride, *via* azide **178**. Azide **178** was prepared from 2-chloro-1-(3-indolyl)ethanone (**177**) and sodium azide in acetone-water at 50 °C (Roy et al., 2006). The resulting azide **178** was reduced to the corresponding amine **179** using H$_2$/Pd in presence of HCl. Condensation of amine **179** with ethyl formate followed by thiation using Lawesson's reagent and cyclization yielded the camalexin isomer **170** in 40% overall yield (**Scheme 2.2**) (Pedras and Abdoli, 2013).
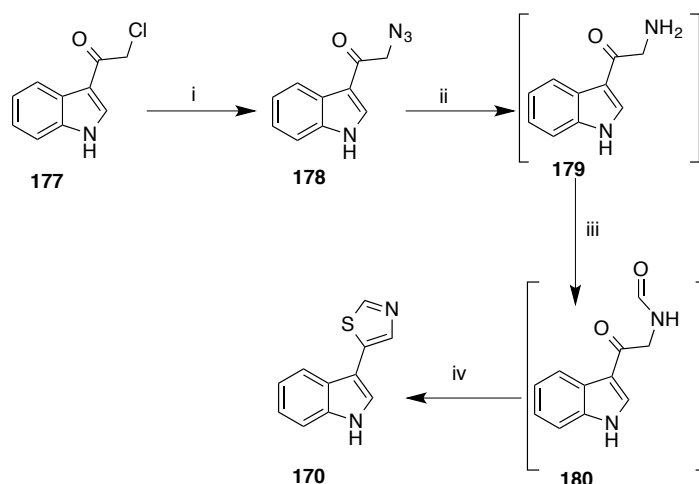
Scheme 2.2   Synthesis of camalexin isomer **170**. Reagents and conditions: (i) NaN_3, H_2O, acetone, 50 °C, 20 h, 81%; (ii) Pd/C, H_2, HCl, MeOH, r.t., 3 h, 85%; (iii) Ethyl formate, Et_3N, THF, 50 °C, 8 h, 86%; (iv) Lawesson's reagent, 1,4-dioxane, 120 °C, 30 min, 68%.

Camalexin isomer **168** was obtained following a procedure used for the synthesis of 4-arylisothiazoles (Pedras and Suchy, 2006). Enol-aldehyde **182** was obtained from transformation of indole-3-acetic acid (**181**) with $POCl_3$/DMF followed by hydrolysis in NaOH solution. Chlorination of enol-aldehyde **182** with thionyl chloride followed by condensation with ammonium thiocyanate yielded thiazolyl-3-indole **168** in 46% overall yield (**Scheme 2.3**).
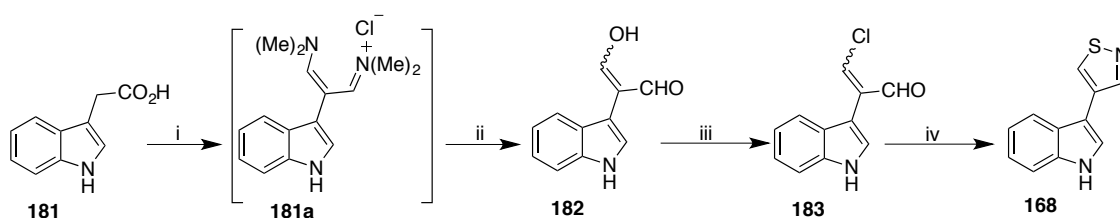
Scheme 2.3   Synthesis of camalexin isomer **168.** Reagents and conditions: (i) $POCl_3$, DMF, 0-90 °C, 3 h, 91%; (ii) NaOH, 1,4-dioxane, reflux, 8 h, 90%; (iii) $SOCl_2$, THF, -20 °C, 10 min; (iv) NH_4SCN, DMF, 70 °C, 12 h, 56%.

The antifungal activities of camalexins **1, 2, 3** and related structures **167-174** were determined against *A. brassicicola* employing a mycelial radial growth assay (potato dextrose agar, PDA), as described in the experimental chapter **Section 3.2**. The antifungal activity was

determined using different concentrations (0.50, 0.20, 0.10 and 0.050 mM) of each compound in PDA. The results of these assays are shown in **Table 2.1**.

Table 2.1   Antifungal activity of camalexins **1**, **2**, **3** and related structures **167-174** against *Alternaria brassicicola.*

Compound (#)	Inhibition ± SD (%)[a]			
	0.50mM	0.20 mM	0.10 mM	0.050 mM
Camalexin (**1**)	-	100 ± 0^b	$76 \pm 0^{b,c,d}$	25 ± 5^d
1-Methycamalexin (**2**)	-	100 ± 0^b	$71 \pm 1^{c,d}$	$43 \pm 3^{b,c}$
6-Methoxycamalexin (**3**)	100 ± 0^b	71 ± 0^d	$28 \pm 5^{g,h}$	14 ± 5^e
4′-Methylcamalexin (**173**)	-	100 ± 0^b	80 ± 0^b	47 ± 3^b
5′-Methylcamalexin (**174**)	-	100 ± 0^b	65 ± 2^d	37 ± 3^c
1*H*-3-(4′-Thiazolyl)indole (**169**)	100 ± 0^b	45 ± 3^f	$33 \pm 5^{f,g}$	n. d.
1*H*-3-(5′-Thiazolyl)indole (**170**)	-	100 ± 0^e	53 ± 5^e	23 ± 2^d
1*H*-3-(4′-Isothiazolyl)indole (**168**)	100 ± 0^b	46 ± 2^f	23 ± 2^h	n. d.
1*H*-3-(2′-Oxazolyl)indole (**167**)	100 ± 0^b	60 ± 0^e	38 ± 1^f	n. d.
1*H*-3-(2′-Thia-3′,4′-diazolyl)indole (**172**)	100 ± 0^b	92 ± 1^c	$72 \pm 1^{b,c,d}$	n. d.
1*H*-3-(2′-Oxa-3′,4′-diazolyl)indole (**171**)	53 ± 1^c	30 ± 3^g	24 ± 1^h	n. d.

[a]The percentage of inhibition was calculated using the formula: % inhibition = 100 - [(growth on amended/growth in control) × 100]; values are averages of three independent experiments conducted in triplicate; n. d. = not determined. For statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted α set at 0.05; n = 3; different letters in the same column ([b]–[h]) indicate significant differences (P < 0.05).

Results of the antifungal assays indicated that camalexins and related structures are inhibitory against *A. brassicicola* at 0.50 mM (**Table 2.1**). At the highest concentration (0.50 mM) all tested compounds could completely inhibit the mycelial growth of *A. brassicicola*, except for 1*H*-3-(2′-oxa-3′, 4′-diazolyl)indole (**171**) that showed only 53% growth inhibition. 1-

Methylcamalexin (**2**) and camalexin (**1**) completely inhibited the mycelial growth of *A. brassicicola* at 0.20 mM; at similar concentration (0.20 mM) 6-methoxycamalexin (**3**) showed 71% inhibition.

Amongst related structures, 4′-methylcamalexin (**173**), 5′-methylcamalexin (**174**) and compound **170** were more inhibitory of fungal growth than camalexin (**1**). Compared to camalexin (**1**), compounds **168**, **171** and **172** were weaker inhibitors against mycelial growth of *A. brassicicola* (Pedras and Abdoli, 2013).

2.1.1.2 *Biotransformation of camalexins*

The transformation of camalexins **1**, **2**, and **3** by *A. brassicicola* was investigated (Pedras and Abdoli, 2013). Due to the inhibitory activity of camalexins **1**, **2** and **3** against *A. brassicicola* (**Table 2.1**), the metabolic studies were carried out using lower concentrations (0.05 mM) of camalexins, analogues and its structural isomers. Cultures were incubated for different periods of times and samples were collected from each culture immediately after addition of phytoalexins up to 10 days. Samples of broth of cultures were extracted and analyzed by HPLC-DAD-ESI-MS. Control solutions containing camalexins (no fungus) or related structures were investigated similarly.
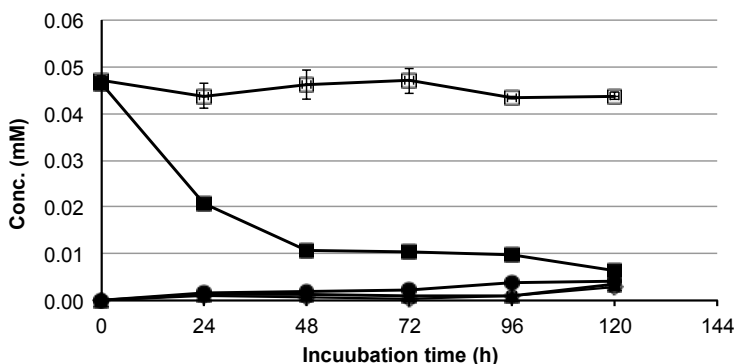
Figure 2.3   Progress curves of transformation of camalexin (**1**, ■) by *Alternaria brassicicola* and formation of products **82** (▲), **83** (◆) and **84** (●) and recovery of camalexin (**1**, □) in control medium.

The HPLC chromatograms of the neutral extracts of cultures of *A. brassicicola* incubated with camalexin (**1**) showed that it was transformed slowly (**Figure 2.3**). The chromatograms of the neutral extracts showed the presence of camalexin (**1**) and additional peaks at 3.6, 4.7 and 7.8 min. New peaks in the neutral extracts were due to indole-3-thiocarboxamide (**82**, t_R = 4.6 min), indole-3-carbonitrile (**83**, t_R = 7.8 min) and indole-3-carboxylic acid (**84**, t_R = 3.6 min), as determined by direct comparison with authentic synthetic samples (nitrile **83** and acid **84** were commercially available) (**Figure 2.4**). No additional peaks were detected either in acidic/basic extracts or in controls.
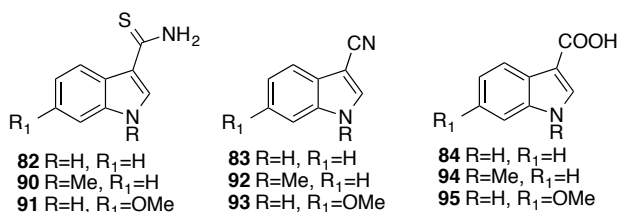
Figure 2.4 Structures of thiocarboxamides **82**, **90**, **91**, nitriles **83**, **92**, **93** and acids **84**, **94**, **95** from transformations of camalexin (**1**), 1-methylcamalexin (**2**) and 6-methoxycamalexin (**3**) by *A. brassicicola.*

To establish the sequence of steps of camalexin (**1**) transformation, each metabolite was administered separately to cultures of *A. brassicicola*. The cultures were incubated, extracted and analyzed by HPLC-DAD-ESI-MS as described for camalexin (**1**). The HPLC chromatograms of the neutral extracts of the cultures incubated with indole-3-thiocarboxamide (**82**) showed that it was transformed to indole-3-carbonitrile (**83**), which in turn was slowly transformed to indole-3-carboxylic acid (**84**) (**Figure 2.5**).
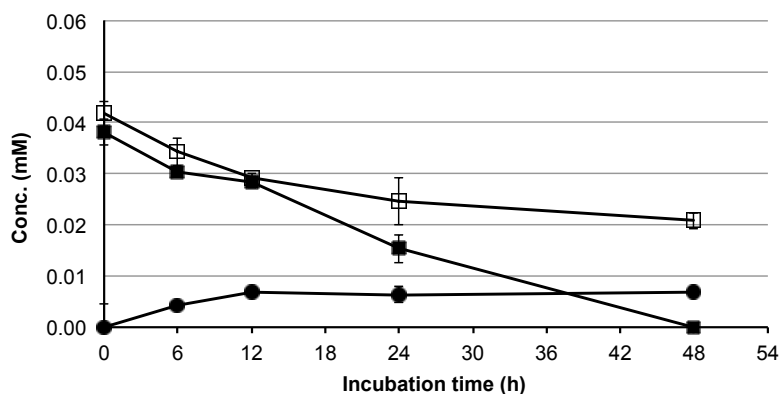
Figure 2.5 Progress curves of transformation of indole-3-thiocarboxamide (**82**, ■) by *Alternaria brassicicola* and formation of product **83** (●) and recovery of indole-3-thiocarboxamide (**82**, □) in control medium.

As previously reported, thiocarboxamide **82** was not stable in media yielding the corresponding nitrile **83** and acid **84** (Pedras et al., 2011a); however, its transformation in cultures was much faster, indicating that an enzyme-mediated transformation occurred

simultaneously (**Figure 2.5**). The HPLC chromatograms of the neutral extracts of the cultures incubated with indole-3-carbonitrile (**83**) showed that it was transformed to indole-3-carboxylic acid (**84**). Indole-3-carboxylic acid (**84**) was stable in cultures of *A. brassicicola* (Pedras and Abdoli, 2013).

Similar biotransformation experiments using 1-methylcamalexin (**2**) and 6-methoxycamalexin (**3**) showed that they were metabolized at similar rate *via* similar intermediates by *A. brassicicola*. Camalexins **2** and **3** were metabolized to corresponding carboxylic acid **94** and **95** *via* thiocarboxamides **90** and **91** and nitriles **92** and **93** (**Figure 2.4**, **Figure 2.6** and **Figure 2.7**).
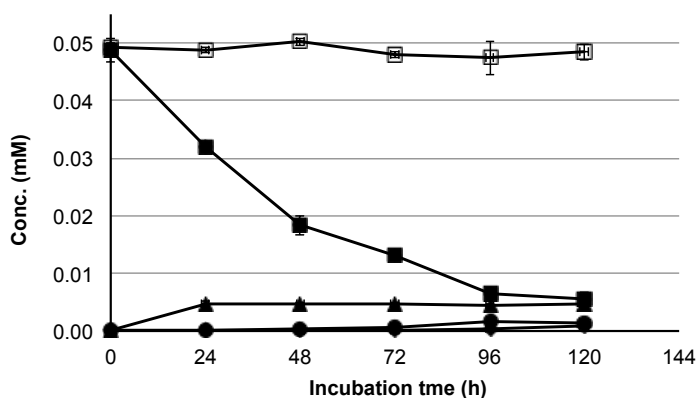
Figure 2.6   Progress curves of transformation of 1-methylcamalexin (**2**, ■) by *Alternaria brassicicola* and formation of products **90** (▲), **92** (◆) and **94** (●) and recovery of 1-methylcamalexin (**2**, □) in control medium.
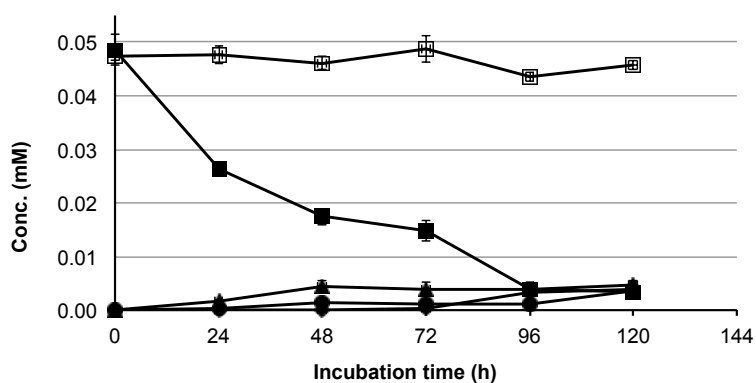
Figure 2.7 Progress curves of transformation of 6-methoxycamalexin (**3**, ■) by *Alternaria brassicicola* and formation of products **91** (▲), **93** (◆) and **95** (●) and recovery of 6-methoxycamalexin (**3**, □) in control medium.

The HPLC chromatograms of the neutral extracts of the MM incubated with tiocarboxamides **82**, **90** and **91** showed their corresponding nitriles and acids (**Figure 2.5**, **Figure 2.8** and **Figure 2.9**).
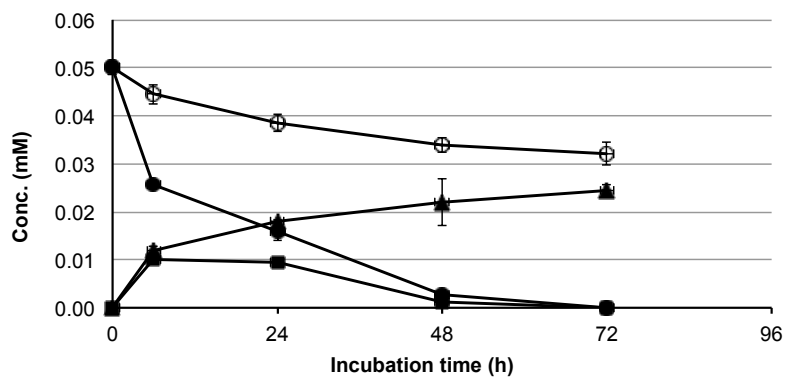
Figure 2.8 Progress curves of transformation of 1-methylindole-3-thiocarboxamide (**90**, ●) by *Alternaria brassicicola* and formation of products **92** (■) and **94** (▲) and recovery of 1-methylindole-3-thiocarboxamide (**90**, ○) in control medium.

50
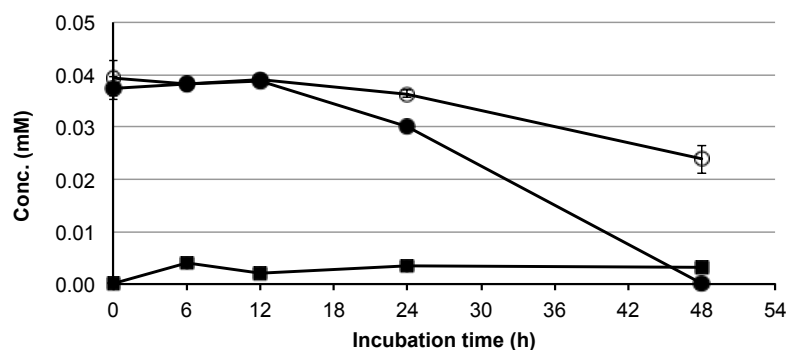
Figure 2.9 Progress curves of transformation of 6-methoxyindole-3-thiocarboxamide (**91**, ●) by *Alternaria brassicicola* and formation of product **93** (■) and recovery of 6-methoxyindole-3-thiocarboxamide (**91**, ○) in control medium.

The HPLC chromatograms of the neutral extracts of cultures of *A. brassicicola* incubated with thiocarboxamides **82**, **90** and **91** showed that they were metabolized at similar rate, while, thiocarboxamide **90** showed higher stability in the controls than the other two thiocarboxamides (ca. 60% left at 48 h after incubation compared to 40%).

2.1.1.3 *Biotransformation of related structures*

To obtain potential intermediate(s) of the degradation pathway of camalexins **1**, **2** and **3** (thiazole ring) by *A. brassicicola*, analogues of camalexin were designed to contain a methyl group at C-4′ or C-5′ of thiazole ring. Compounds **173** and **174** were synthesized as summarized in **Section 2.1.1.1** and incubated with cultures of *A. brassicicola* and also un-inoculated (control) medium at 0.050 mM, separately. Samples were collected at different periods of times, extracted and analyzed by HPLC-DAD-ESI-MS, as described for camalexin (**1**) (Pedras and Abdoli, 2013). HPLC analysis of the broth extracts of cultures incubated with analogues **173** and **174** indicated that both compounds were transformed completely by *A. brassicicola* at a rate slightly faster than metabolism of camalexin (**1**) (**Figure 2.3**). Time-course analyses revealed that, 4′-methylcamalexin (**173**) was completely transformed within 96 h; a new peak was detected at 10.3 min, with a UV spectrum different from the metabolites of camalexin (**1**). The metabolite at 10.3 min was isolated from larger scale cultures of *A. brassicicola* incubated with 4′-

methylcamalexin (**173**) as described in **Section 3.4.1.2**. Analyses of spectroscopic data allowed the identification of the metabolite with $t_R = 10.3$ min as (2-(1H-indol-3-yl)thiazol-4-yl)methnol (**185**) (**Figure 2.12**). Metabolite **185** was not detected either in control cultures or in media incubated with compound **173**. No additional compounds were detected in either acidic/basic extracts of any cultures or controls. HPLC analysis of the cultures incubated with compound **185** did not show any metabolites. Also, recovery of compound **185** from the cultures and controls were comparable, which revealed that compound **185** was not transformed by *A. brassicicola* and remained intact in both cultures and media up to 72 h (**Figure 2.11**).
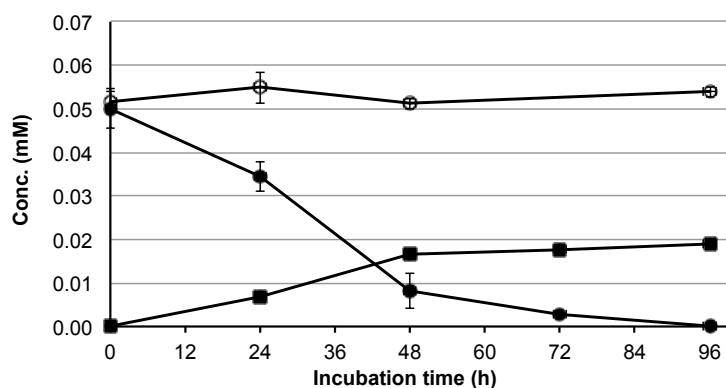
Figure 2.10   Progress curves of transformation of 4′-methylcamalexin (**173**, ●) by *Alternaria brassicicola* and formation of product **185** (■) and recovery of 2-(1H-indol-3-yl)-4-methylthiazole (**173**, ○) in control medium.
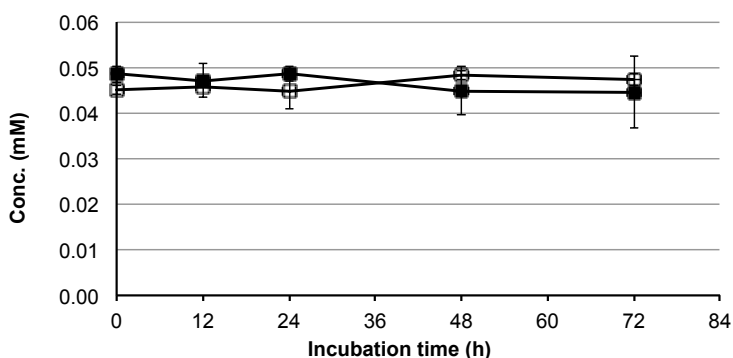
Figure 2.11 Curves representing the recovery of (2-(1*H*-indol-3-yl)thiazol-4-yl)methnol (**185**, ■) in *Alternaria brassicicola* and in control medium (□).

184　　　**185**
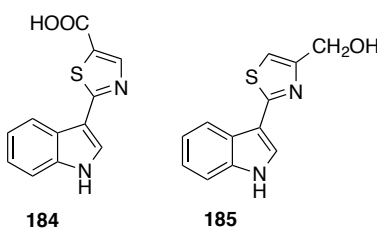
Figure 2.12 Structures of (2-(1*H*-indol-3-yl)thiazol-4-yl)methnol (**185**) and (2-(1*H*-indol-3-yl)thiazol-5-carboxylic acid (**184**) from transformations of compounds **173** and **174** by *Alternaria brassicicola.*

Time-course studies revealed that 5′-methylcamalexin (**174**) was almost completely metabolized within 96 h by *A. brassicicola* at a rate comparable to 4′-methylcamalexin (**173**) (**Figure 2.13**), but no metabolites could be detected in the extracts of cultures incubated with **174**. Cultures of *A. brassicicola* were prepared in water and were fed with 5′-methylcamalexin (**174**) as described in **Section 3.4.1.2**. Samples were collected at various times, concentrated to dryness using a freeze-dryer and the residue was analyzed directly by HPLC-DAD-ESI-MS. HPLC-ESI-MS data of the freeze-dried samples of cultures indicated the presence of a highly polar metabolite. The HPLC-ESI-MS (negative mode) analysis of the new metabolite indicated an ion at *m/z* 243 [M-H]⁻, which was 32 units higher than compound **174**. Based on these ions the metabolite was proposed to contain two additional oxygen atoms. (2-(1*H*-indol-3-yl)thiazol-5-

carboxylic acid (**184**) (**Figure 2.12**) was proposed to result from oxidation of compound **174** as determined by direct comparison with an authentic synthetic sample. Acid **184** was not soluble in ethyl acetate, therefore was not detected in extracts of any cultures. Compound **184** was not detected either in control cultures or in media incubated with compound **174**. No additional compounds were detected in either acidic/basic extracts of any cultures or controls. Acid **184** was not metabolized by *A. brassicicola* and remained intact in cultures for at least 120 h.
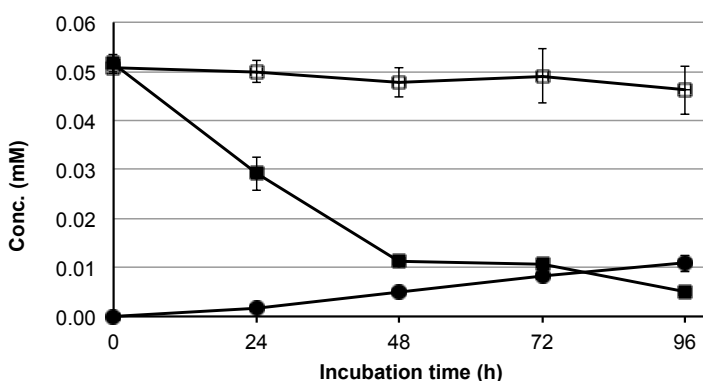
Figure 2.13   Progress curves of transformation of 5′-methylcamalexin (**174**, ■) by *Alternaria brassicicola* and formation of product **184** (●) and recovery of 2-(1*H*-indol-3-yl)-5-methylthiazole (**174**, □) in control medium.

Compounds **169**, **170**, **168**, **167**, **172** and **171** were designed to probe the selectivity of the oxidative enzyme(s) of *A. brassicicola* in degradation of thiazol rings of camalexins (Pedras and Abdoli, 2013). HPLC analyses of broth extracts of cultures of *A. brassicicola* incubated with 1*H*-3-(2′-oxazolyl)indole (**167**) and 2-(1*H*-indol-3-yl)-1,3,4-oxadiazole (**171**) separately, indicated that these analogues were transformed by *A. brassicicola* slowly (>120 h, **Figure 2.15**, **Figure 2.16**) to a metabolite with t_R = 3.6 min; the structure of this metabolite was indole-3-carboxamide (**186**) (**Figure 2.14**), as determined by direct comparison with an authentic synthetic sample. Indole-3-carboxamide (**186**) was not detected either in control cultures or in media incubated with compounds **167** or **171**. No other metabolites were detected in acidic/basic extracts of either culture. Indole-3-carboxamide (**186**) remained stable against transformation by *A. brassicicola*.

186
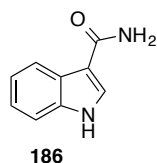
Figure 2.14 Structure of indole-3-carboxamide (**186**) from transformation of 2-(1*H*-indol-3-yl)-1,3,4-oxadiazole (**171**) by *Alternaria brassicicola*.

Figure 2.15 Progress curves of transformation of 1*H*-3-(2′-oxazolyl)indole (**167**, ●) by *Alternaria brassicicola* and formation of 1H-indole-3-carboxamide (**186**, ■) and recovery of 1*H*-3-(2′-oxazolyl)indole (**167**, ○) in control medium.
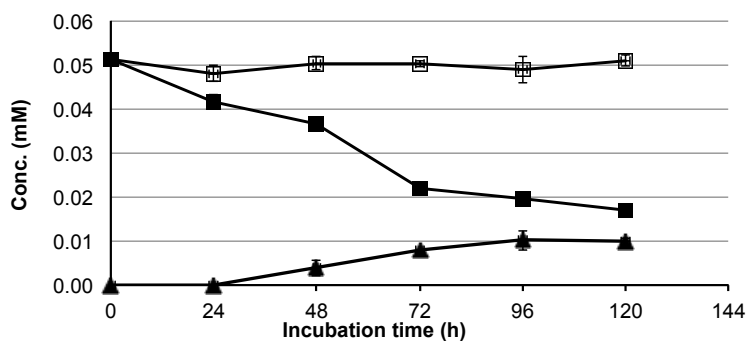
Figure 2.16 Progress curves of transformation of 2-(1*H*-indol-3-yl)-1,3,4-oxadiazole (**171**, ■) by *Alternaria brassicicola* and formation of 1*H*-indole-3-carboxamide (**186**, ▲) and recovery of 2-(1*H*-indol-3-yl)-1,3,4-oxadiazole (**171**, □) in control medium.

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The HPLC-DAD-ESI-MS chromatograms of neutral extracts of cultures of *A. brassicicola* incubated with 1*H*-3-(2′-thia-3′,4′-diazolyl)indole (**172**) showed that this compound was transformed slowly (>120 h, **Figure 2.17**) by *A. brassicicola* to indole-3-thiocarboxamide (**82**), which was transformed further to indole-3-carboxylic acid (**84**) *via* indole-3-carbonitrile (**83**) (Pedras and Abdoli, 2013). None of these metabolites were detected either in control cultures or in media incubated with compound **172**. No other metabolites were detected in acidic or basic extracts of either culture. 1*H*-3-(2′-thia-3′,4′-diazolyl)indole (**172**) was metabolized by *A. brassicicola* with the transformation pathway identical to camalexin (**1**), however the rate of transformation was slower than camalexin (**1**).
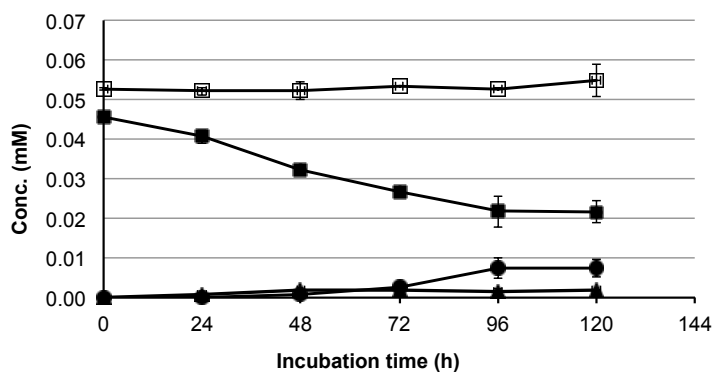
Figure 2.17   Progress curves of transformation of 1*H*-3-(2′-thia-3′,4′-diazolyl)indole (**172**, ■) by *Alternaria brassicicola* and formation of indole-3-thiocarboxamide (**82**, ●) and indole-3-carboxilic acid (**84**, ▲) and recovery of 1*H*-3-(2′-thia-3′,4′-diazolyl)indole (**172**, □) in control medium.

HPLC analysis of neutral, basic and acidic extracts of cultures of *A. brassicicola* incubated with 1*H*-3-(4′-thiazolyl)indole (**169**), 1*H*-3-(5′-thiazolyl)indole (**170**) and 1*H*-3-(4′-isothiazolyl)indole (**168**) revealed that these compounds were extracted under neutral condition and no additional compounds were detected. Also, recovery of compounds **169**, **170** and **168** from culture and control samples was comparable (**Figure 2.18**, **Figure 2.19** and **Figure 2.20**).
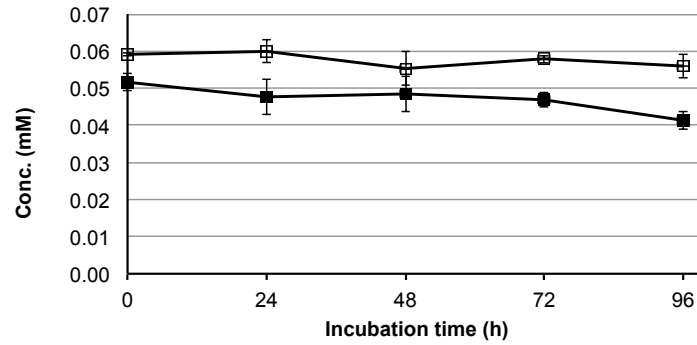
Figure 2.18 Curves representing the recovery of **168** in cultures of *Alternaria brassicicola* in minimal medium (■) and in control medium (□).
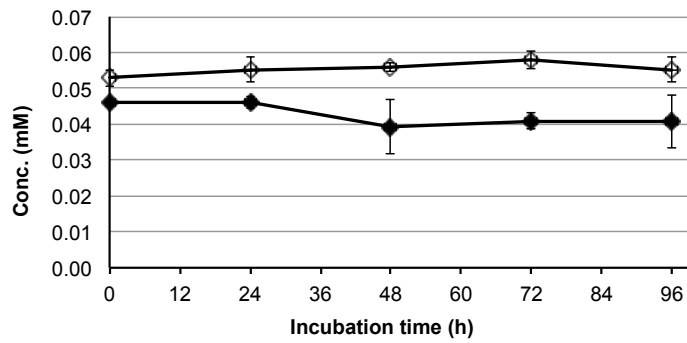
Figure 2.19 Curves representing the recovery of **169** in cultures of *Alternaria brassicicola* in minimal medium (◆) and in control medium (◇).
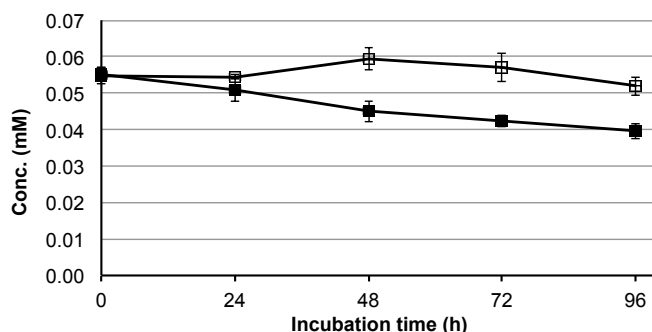
Figure 2.20   Curves representing the recovery of **170** in cultures of *Alternaria brassicicola* in minimal medium (■) and in control medium (□).

To better understand the degradation pathway of thiazolyl rings of camalexins by *A. brassicicola*, it was important to isolate potential precursor(s) of indole-3-thiocarboxamide (**82**) from the cultures of *A. brassicicola* incubated with camalexin (**1**). For this reason transformation of camalexin (**1**) by *A. brassicicola* was investigated under different conditions. For example, cultures of *A. brassicicola* were incubated at higher concentration of camalexin (**1**) (0.10 mM *vs* 0.050 mM), at higher temperature (30 °C *vs* 23 °C) or in different media (PDB *vs* chemically modified media). However, HPLC-DAD-ESI-MS of any of these conditions showed no additional camalexins metabolites. It is also possible that precursor(s) of indole-3-thiocarboxamide (**82**) are more polar than amide **82** and are not extractable from the culture with EtOAc. Cultures of *A. brassicicola* in water were incubated with camalexin (0.05 mM). Samples were collected at different periods. Mycelia were filtered off and the filtrate was extracted with EtOAc then the extracted aqueous was freeze-dried. Both EtOAc extracts and the aqueous residue were analyzed with HPLC-DAD-ESI-MS. HPLC chromatograms of ethyl acetate extracts showed thiocarboxamide, nitrile and acid from transformation of camalexin (**1**) and no more metabolites were detected in the aqueous residue (Pedras and Abdoli, 2013).

2.1.1.4   Synthesis and antifungal activity of metabolites

Thiocarboxamides **82**, **91** and **90** that resulted from transformation of camalexins **1**, **2** and **3** were obtained by reaction of their corresponding nitriles **83**, **92** and **93** with thioacetamide in 10% HCl-DMF according to previously published procedures (Gu et al., 1999; Pedras et al., 2011a) as described in **Sections 3.4.1.3.1** and **3.4.1.3.2**. 6-Methoxyindole-3-carbonitrile (**93**) was synthesized in reasonable yield from dehydration of oxime **188** using acetic anhydride in presence of pyridine under reflux conditions (Pedras et al., 2010b) and then was transformed to thiocarboxamide **91** as described above in 38% overall yield (**Scheme 2.4**) (Gu et al., 1999; Pedras et al., 2011a; Pedras and Abdoli, 2013).
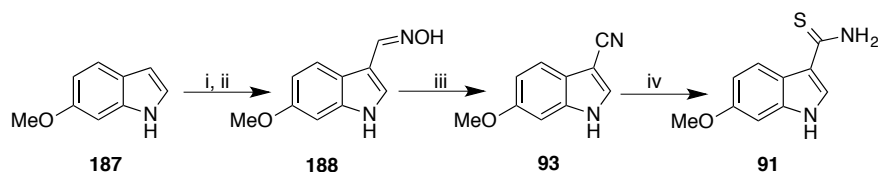
Scheme 2.4   Syntheses of **93** and **91**. Reagents and conditions: (i) $POCl_3$, DMF, r.t., 2.5 h, 98%; (ii) $HONH_2 \cdot HCl$, NaOAc, 95% EtOH, H_2O, r.t., 3 h; (iii) Ac_2O, pyridine, CH_2Cl_2, reflux, 9 h, 96%; (iv) Thioacetamide, HCl/DMF, 90 °C, 12 h, 40%.

1-Methylindole-3-carboxylic acid (**94**) and 6-methoxyindole-3-carboxylic acid (**95**) were synthesized by oxidation of 1-methylindole-3-carboxaldehyde (**189**) and 6-methoxyindole-3-carboxaldehyde (**190**) respectively using sodium chlorite ($NaClO_2$) under mild acidic condition (**Scheme 2.5**) as described in **Section 3.4.1.3.4** (Pedras and Abdoli, 2013).

189 R=Me, R1=H
190 R=H, R1=OMe

94 R=Me, R1=H
95 R=H, R1=OMe
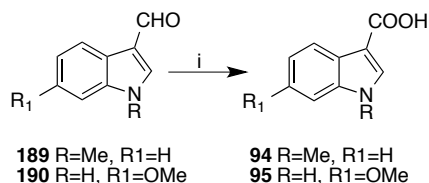
Scheme 2.5   Syntheses of acids **94** and **95**. Reagents and conditions: (i) $NaClO_2$, NaH_2PO_4, *t*-butanol, 2-methylbut-2-ene, H_2O, 14 h, r.t., 75% (**94**) and 60 h, 52% (**95**).

The new compounds 1*H*-3-(4-hydroxymethyl-2-thiazolyl)indole (**185**) and 2-(1*H*-indol-3-yl)-4-thiazolecarboxylic acid (**184**) were synthesized as summarized in **Scheme 2.6**. Ester **191** was prepared by condensation of thiocarboxamide **82** with ethyl bromopyruvate in ethanol under reflux (Moody et al., 1997) and then it was reduced to alcohol **185** using LiAlH$_4$ in 83% yield (**Scheme 2.6**). Carboxylic acid **184** was synthesized by condensation of thiocarboxamide **82** with methyl 2-chloro-3-oxopropanoate (Gangjee et al., 2001) in ethanol under reflux, followed by basic hydrolysis of resulting methyl ester (**192**) in 62% overall yield (**Scheme 2.6**).
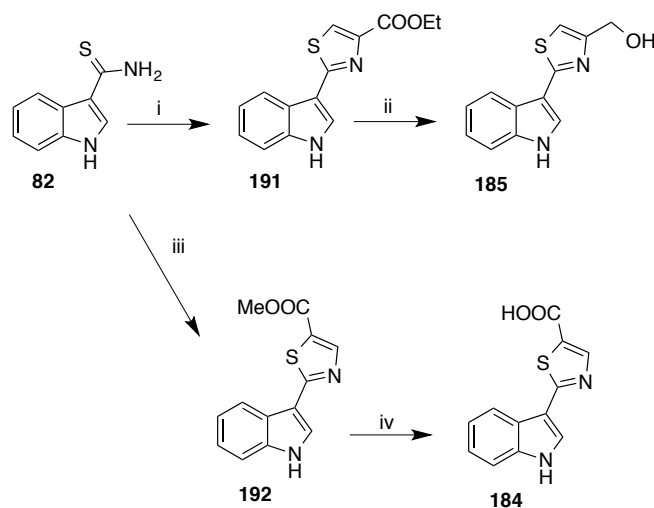
Scheme 2.6   Syntheses of compounds **185** and **184**. Reagents and conditions: (i) Ethyl bromopyruvate, 95% EtOH, reflux, 1 h; (ii) LiAlH$_4$, THF, 10 h, 83% from **82**; (iii) Methyl 2-chloro-3-oxopropanoate, 95% EtOH, reflux, 5 h, 62%; (iv) NaOH, THF, reflux, 4 h, 100%.

To establish if the transformation of camalexins **1**, **2**, **3** and related structures **167** and **171-174** by *A. brassicicola* were detoxifications, mycelial radial growth assays were carried out. The antifungal activities against *A. brassicicola* of all metabolites were determined as described in **Section 3.2**; results of these assays are shown in **Table 2.2**.

Table 2.2 Antifungal activity of compounds **82-84**, **90-95** and **184-186** against *Alternaria brassicicola*.

Compound (#)	Inhibition ± SD (%)[a]		
	0.50 mM	0.20 mM	0.10 mM
Indole-3-thiocarboxamide (**82**)	53 ± 3^f	31 ± 3^{ef}	24 ± 3^{cd}
Indole-3-carbonitrile (**83**)	100 ± 0^b	92 ± 1^b	73 ± 3^b
Indole-3-carboxylic acid (**84**)	26 ± 2^h	11 ± 2^i	n. i.
1-Methylindole-3-thiocarboxamide (**90**)	60 ± 3^e	35 ± 3^{de}	21 ± 3^{de}
6-Methoxyindole-3-thiocarboxamide (**91**)	40 ± 3^g	21 ± 3^{gh}	n. i.
1-Methylindole-3-carbonitrile (**92**)	100 ± 0^b	35 ± 3^{de}	8 ± 3^f
6-Methoxyindole-3-carbonitrile (**93**)	100 ± 0^b	52 ± 3^c	31 ± 0^c
1-Methylindole-3-carboxylic acid (**94**)	26 ± 2^h	16 ± 2^{hj}	n. i.
6-Methoxyindole-3-carboxylic acid (**95**)	34 ± 0^g	26 ± 2^{fg}	n. i.
2-1*H*-3-(4-Hydroxymethyl-2-thiazolyl)indole (**185**)	90 ± 2^c	41 ± 4^d	13 ± 2^f
2-(1*H*-Indol-3-yl)-4-thiazolecarboxylic acid (**184**)	85 ± 1^c	38 ± 3^{de}	11 ± 2^f
Indole-3-carboxamide (**186**)	68 ± 1^d	41 ± 2^d	15 ± 3^{ef}

[a]The percentage of inhibition was calculated using the formula: % inhibition = 100 - [(growth on amended/growth in control) × 100]; values are averages of three independent experiments conducted in triplicate; n. i. = no inhibition. For statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted α set at 0.05; n = 3; different letters in the same column ([b]–[i]) indicate significant differences ($P < 0.05$).

Comparison of the antifungal activities (**Table 2.2**) suggested that growth inhibition gradually decreased from thiocarboxamides **82**, **90** and **91** to acids **84**, **94** and **95** and the inhibition was weaker than that caused by their parent compounds. However, nitriles **83**, **92** and **93** showed strong inhibitory activity against *A. brassicicola*; at the highest concentration (0.50 mM) of nitriles **83**, **92** and **93,** the mycelial growth of *A. brassicicola* was completely inhibited, while acids **84**, **94** and **95** did not show substantial activity against *A. brassicicola* (**Table 2.2**). Metabolites **184** and **185** resulted from transformation of **173** and **174** showed weaker antifungal

activity against *A. brassicicola* compared to their parent compounds. Results of antifungal activities showed that the transformation of camalexins **1**, **2**, **3** and analogues **167** and **171-174** by *A. brassicicola* are detoxifications (Pedras and Abdoli, 2013).

2.1.1.5 *Discussion and conclusion*

Bioassays with camalexin (**1**), 1-methylcamalexin (**2**), 6-methoxycamalexin (**3**), and related structures **167-174** showed that amongst all camalexins **1**, **2**, **3** and compounds **173** and **174** exhibited strong inhibitory activity against *A. brassicicola* while compound **171** was the weakest (**Table 2.1**). The metabolites resulting from transformations of camalexins and the related structures were much less toxic against *A. brassicicola* compared to their parent compounds (**Table 2.2**) (Pedras and Abdoli, 2013). Results of the antifungal activities indicated that the transformations of camalexin (**1**), 1-methylcamalexin (**2**) and 6-methoxycamalexin (**3**) by *A. brassicicola* are detoxifications (Pedras and Abdoli, 2013).

Camalexin (**1**), 1-methylcamalexin (**2**) and 6-methoxycamalexin (**3**) were metabolized by *A. brassicicola* to thiocarboxamides **82**, **90** and **91** at almost similar rates (ca. 120 h) during enzyme mediated oxidative degradation of their thiazolyl rings (Pedras and Abdoli, 2013). Thiocarboxamides **82**, **90** and **91**were transformed further by *A. brassicicola* to acids **84**, **94** and **95** respectively *via* their corresponding nitriles (**83**, **92** and **93**). The pathway of transformations of camalexins **1**, **2** and **3** by *A. brassicicola* is proposed in **Scheme 2.7**.
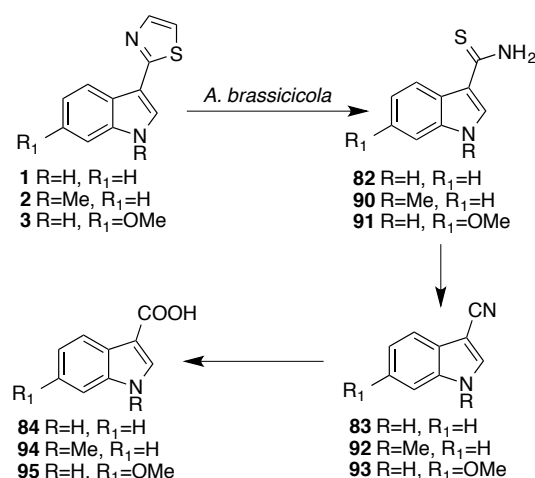
Scheme 2.7 Biotransformations of camalexin (**1**), 1-methylcamalexin (**2**) and 6-methoxycamalexin (**3**) by *Alternaria brassicicola* and metabolic products.

Previous work showed that camalexin (**1**) was transformed to indole-3-thiocarboxamide (**82**) quickly (ca. 12 h) by *B. cinerea* (Pedras and Hossain, 2011). These results indicated that camalexin (**1**) was transformed by *A. brassicicola* to metabolic products that were identical to those observed in cultures of *B. cinerea,* however, the rate of transformation in *B. cinerea* was much faster than *A. brassicicola* (12 h *vs* 10 days) (Pedras and Abdoli, 2013; Pedras and Hossain, 2011). The transformation pathway suggested that enzymatic degradation of thiazolyl ring of camalexin (**1**) to thiocarboxamide **82** is the most important step in camalexin (**1**) detoxification; this step is followed by further transformation of thiocarboxamide **82** to acid **84** *via* nitrile **83** (**Scheme 2.7**). To better understand the mechanism of degradation of thiazolyl ring of camalexin (**1**) by *A. brassicicola* and also to probe the selectivity of the enzyme(s) involving in degradation of the thiazolyl ring of camalexin (**1**), transformations of related structures **167-174** were investigated in cultures of *A. brassicicola*. Related structures **173** and **174** were metabolized to compounds **185** and **184** respectively at similar rate (**Figure 2.21**, $t_{1/2}$ ca. 36 h, complete ca. 96 h) by *A. brassicicola* (Pedras and Abdoli, 2013). Transformations of compounds **173** and **174** by *A. brassicicola* were somewhat faster than camalexin (**1**) (**Figure 2.3**) (Pedras and Abdoli, 2013). Compounds **185** and **184** were not metabolized further by *A. brassicicola*. The transformation of related structures **173** and **174** by *A. brassicicola* is shown in **Scheme 2.8** (Pedras and Abdoli, 2013).
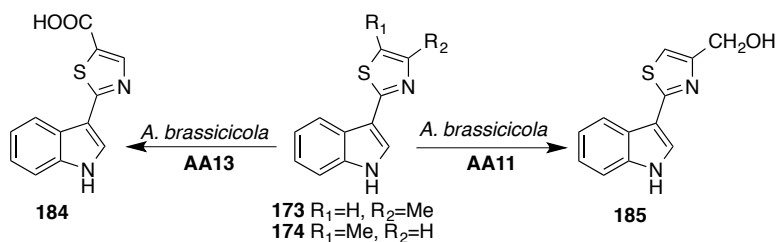
Scheme 2.8 Biotransformations of 1*H*-3-(4-hydroxymethyl-2-thiazolyl)indole (**173**) and 2-(1*H*-indol-3-yl)-4-thiazolecarboxylic acid (**174**) by *Alternaria brassicicola* and metabolic products.
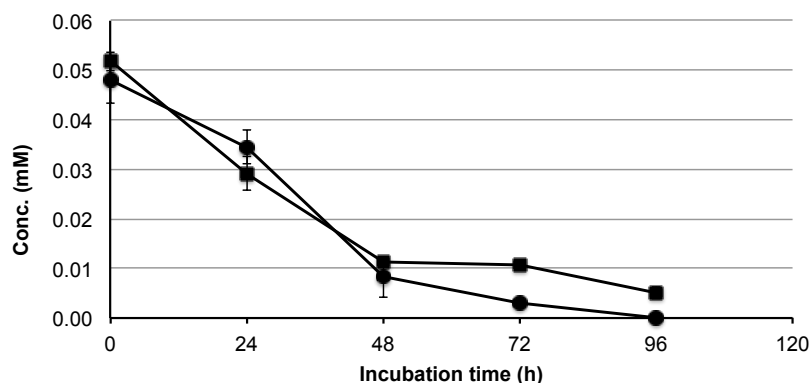
Figure 2.21 Progress curves of transformations of 1*H*-3-(4-hydroxymethyl-2-thiazolyl)indole (**173**) (●) and 2-(1*H*-indol-3-yl)-4-thiazolecarboxylic acid (**174**) (■) by *Alternaria brassicicola*.

The transformations of analogues **173** and **174** revealed that, substitution at C-4′ or C-5′ of thiazolyl rings of compounds **173** and **174** blocked the degradation of the thiazole ring by *A. brassicicola*, however, did not prevent oxidation of methyl group of **173** and **174** (Pedras and Abdoli, 2013). Analogues **167** and **171** were metabolized to carboxamide **186** at similar rate (**Figure 2.22**, $t_{1/2}$ ca. 72 h, complete >120 h) by *A. brassicicola* (Pedras and Abdoli, 2013). Carboxamide **186** did not metabolize further by *A. brassicicola*. The transformations of analogues **167** and **171** by *A. brassicicola* are shown in **Scheme 2.9** (Pedras and Abdoli, 2013).
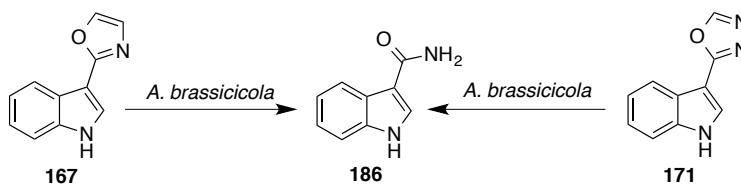
Scheme 2.9   Biotransformations of analogues 1*H*-3-(2′-oxazolyl)indole (**167**) and 1*H*-3–(2′-oxa-3′,4′-diazolyl)indole (**171**) by *Alternaria brassicicola* and metabolic product.

Thiadiazole **172** was metabolized by *A. brassicicola* to thiocarboxamide **82** similar to metabolism of camalexin (**1**). Thiocarboxamide **82** was metabolized further to indole-3-carboxylic acid (**84**) *via* indole-3-carbonitrile (**83**) (Pedras and Abdoli, 2013). The pathway of transformation of compound **172** by *A. brassicicola* is proposed in **Scheme 2.10** (Pedras and Abdoli, 2013).
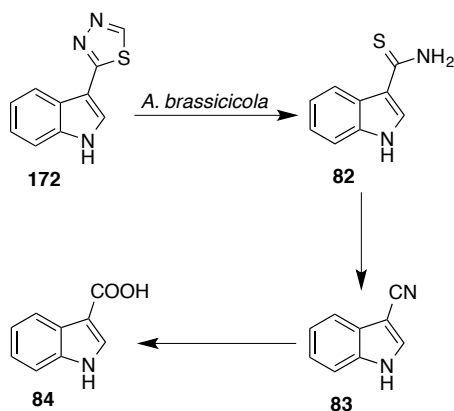
Scheme 2.10 Biotransformation of 1*H*-3-(2′-thia-3′,4′-diazolyl)indole (**172**) by *Alternaria brassicicola* and metabolic products.

The rate of transformations of oxazole **167** and diazoles **172** and **171** by *A. brassicicola* was slower than camalexin (**1**) (**Figure 2.22**, t$_{1/2}$ ca. 72 h, complete >120 h).
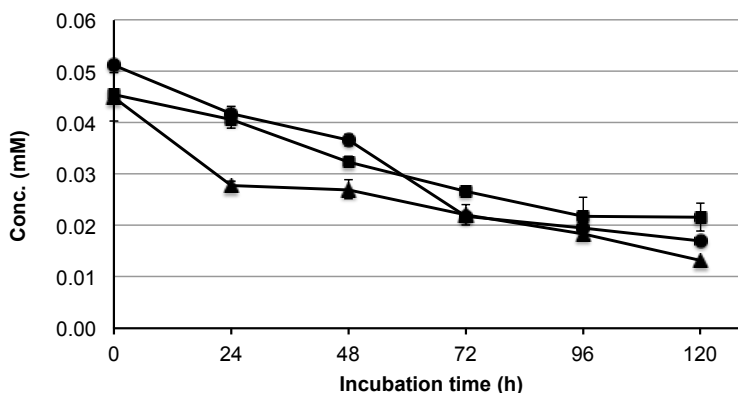
Figure 2.22 Progress curves of transformations of 1*H*-3-(2′-oxazolyl)indole (**167**, ▲), 1*H*-3–(2′-oxa-3′,4′-diazolyl)indole (**171**, ●) and 1*H*-3-(2′-thia-3′,4′-diazolyl)indole (**172**, ■) by *Alternaria brassicicola*.

Camalexins **168**, **169** and **170** were resistant to metabolism by *A. brassicicola*, comparing that with transformations of camalexins **1**, **2**, **3** and compounds **56**, **73**, **74** revealed that oxidation of thiazole ring by *A. brassicicola* required it to be attached to indole by C-2′.

In conclusion, it was shown for the first time that the plant pathogen *A. brassicicola* was able to slowly detoxify camalexins **1**, **2** and **3** to the corresponding thiocarboxamides **82**, **90** and **91** using similar transformation pathways, involving an oxidative degradation of thiazolyl rings of camalexins. Thiocarboxamides **82**, **90** and **91**were metabolized further to the corresponding acids, the least antifungal compounds, via nitriles **83**, **92** and **93**. Transformations of analogues **173** and **174** by *A. brassicicola* were faster than camalexins but without degradation of the thiazole ring. That showed, methyl group on either C-4′ or C-5′ of compounds **173** and **174** changed the transformation pathways of these compound but did not prevent the metabolisms. Furthermore, structural isomers **168**, **169** and **170** were not metabolized by *A. brassicicola*, thus it can be concluded that the degradation of thiazole ring by *A. brassicicola* requires it to attach to indole by C-2′.

Detoxification of camalexins **1**, **2**, and **3** by *A. brassicicola* is slow in comparison with metabolism of other phytoalexins like brassinin (ca. 12 h at 0.10 mM) (Pedras et al., 2009a) by this fungus. This slow detoxification of camalexin (**1**) is consistent with its importance in the resistance of *A. thaliana* to *A. brassicicola* (Thomma et al., 1999). According to the high

antifungal activity of camalexins **1**, **2**, and **3** against *A. brassicicola* as well as slow transformation of them by *A. brassicicola,* engineering the biosynthetic pathway of camalexins into cultivated *Brassica* species is one of the assured ways to protect cultivated crucifers against black spot diseases.

2.1.2 Cyclobrassinin and 1-methylcyclobrassinin

Cyclobrassinin (**4**) is a phytoalexin produced mainly by *Brassica species* and one of the first cruciferous phytoalexin isolated from Chinese cabbage (Pedras et al., 2011b). Cyclobrassinin (**4**) is a biosynthetic precursor of some cruciferous phytoalexins such as brassilexin (**7**) and rutalexin (**5**) (Pedras et al., 2011b). 1-Methylcyclobrassin (**193**) is not a natural product; it was prepared by methylation of cyclobrassinin (**4**).

4 R=H
193 R=CH₃
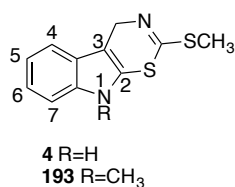
Figure 2.23   Structures of cyclobrassinin (**4**) and 1-methylcyclobrassinin (**193**).

2.1.2.1 Synthesis and antifungal activity

Cyclobrassinin (**4**) and 1-methylcyclobrassinin (**193**) were synthesized following a published procedure (Pedras et al., 2011b), as described in **Section 3.3.3**. The antifungal activities of cyclobrassinin (**4**) and 1-methylcyclobrassinin (**193**) were determined against *A. brassicicola* employing a mycelial radial growth assay (potato dextrose agar, PDA), as described in the **Section 3.2**. Three different concentrations (0.50, 0.20 and 0.10 mM) of each compound in PDA were used for determination of the antifungal activity. Results of these assays are shown in **Table 2.3**.

Table 2.3 Antifungal activity of compounds **4** and **193** against *Alternaria brassicicola*.

Compound (#)	Inhibition ± SD (%)[a]		
	0.50 mM	0.20 mM	0.10 mM
Cyclobrassinin (**4**)	39 ± 3[c]	25 ± 3[d]	18 ± 5[e]
1-Methylcyclobrassinin (**193**)	69 ± 3[b]	29 ± 5[d]	16 ± 3[e]

[a] The percentage of inhibition was calculated using the formula: % inhibition = 100 − [(growth on amended/growth in control) × 100]; values are averages of three independent experiments conducted in triplicate. For statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted α set at 0.05; n = 6; different letters ([b]–[e]) indicate significant differences (P < 0.05).

Results of the antifungal assays indicated that at the highest tested concentration (0.50 mM) cyclobrassinin (**4**) showed about 40% inhibition on mycelial growth of *A. brassicicola*. 1-Methylcyclobrassinin (**193**) caused 69% inhibition at 0.50 mM (**Table 2.3**), however, at low concentration (0.20 and 0.10 mM) both compounds behaved similarly and showed similar inhibitory activity against mycelial growth of *A. brassicicola* (**Table 2.3**).

2.1.2.2 *Biotransformation of cyclobrassinin and 1-methylcyclobrassinin*

The transformations of cyclobrassinin (**4**) and 1-methylcyclobrassinin (**193**) by *A. brassicicola* were investigated. These experiments were carried out using 0.10 mM of cyclobrassinin (**4**) and 1-methylcyclobrassinin (**193**) (Pedras et al., 2013; Pedras and Minic, 2014). Cultures were incubated for different periods of time and samples were collected from each culture immediately after addition of cyclobrassinin (**4**). Samples of cultures were extracted and analyzed by HPLC-DAD-ESI-MS. Control solutions containing cyclobrassinin (**4**) and 1-methylcyclobrassinin (**193**) (only MM, no fungus) were investigated similarly. Results of preliminary time-course studies were somewhat inconsistent; the amounts of cyclobrassinin (**4**) or 1-methylcyclobrassinin (**193**) in triplicate samples varied by ca. 30% (poor recovery of **4** or **193**). To solve this problem cultures of *A. brassicicola* were prepared in MM for each time point (5 ml in 25 ml Erlenmeyer flasks in triplicates) and were incubated with cyclobrassinin (**4**) (**Section 3.4.2.1**). Cultures were filtered, the filtrate was extracted and the flasks and mycelia

were rinsed with EtOAc. The combined EtOAc extracts were concentrated to dryness and the residue was analyzed by HPLC-DAD-ESI-MS. Control solutions containing cyclobrassinin (**4**) (only MM, no fungus) were analyzed similarly to determine its chemical stability during the incubation time.
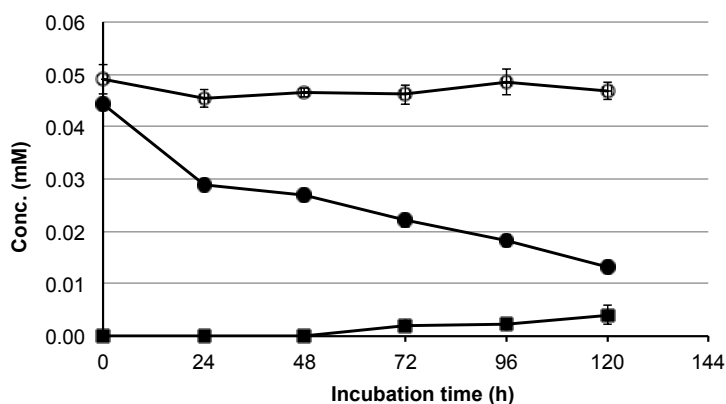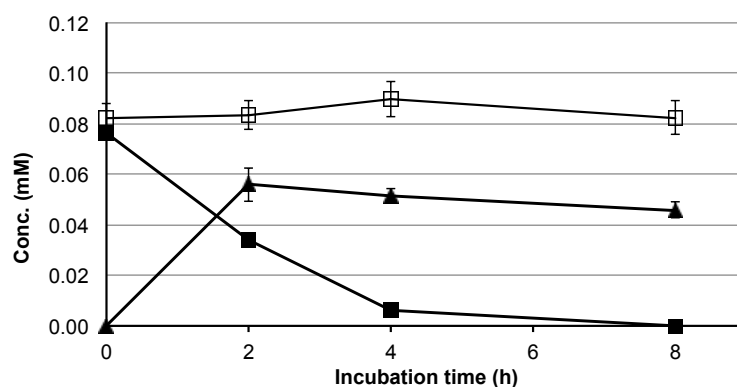

Figure 2.24 Progress curves of transformation of cyclobrassinin (**4**, ■) by *Alternaria brassicicola*, formation of product (**137**, ▲) and recovery of cyclobrassinin (**4**, □) in minimal medium.

The HPLC chromatograms of neutral extracts of cultures of *A. brassicicola* incubated with cyclobrassinin (**4**) showed that it was not detected after 8 h (**Figure 2.24**). The chromatograms of the neutral extracts showed the presence of cyclobrassinin (**4**) and an additional peak at 13.6 min. The metabolite at 13.6 min was obtained from larger-scale cultures but was not sufficient for complete spectroscopic characterization (enough for ^{1}H NMR and HRMS analyses). However, no reasonable MS data was obtained for this compound using available ionization techniques. The ^{1}H NMR of this metabolite showed all signals corresponding to cyclobrassinin (**4**) with slight changes in their chemical shifts. Considering the ^{1}H NMR, structure **137** was proposed for the metabolite at 13.6 min (**Figure 2.25**).
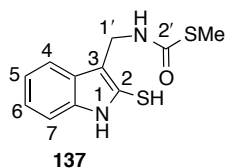
Figure 2.25 Structure of S-methyl [(2-sulfanyl-1*H*-indolyl-3)methyl]carbamothioate (**137**) from transformation of cyclobrassinin (**4**) by *Alternaria brassicicola*.

Compound **137** was synthesized as described in **Section 3.4.2.2.1** and its data was compared with those of the isolated compound, confirming the assigned structure (Pedras et al., 2013). Results of biotransformation of cyclobrassinin (**4**) revealed that compound **137** was metabolized further by *A. brassicicola.* However, no other metabolites were detected in the neutral extracts (**Figure 2.26**). Metabolite **137** was administrated separately to cultures of *A. brassicicola* in water (culture preparation in water described in **Section 3.4.2.1**). Samples were collected at various times, freeze-dried, the residue was dissolved in MeOH-H_2O (1:1) and analyzed directly by HPLC-DAD-ESI-MS. HPLC-ESI-MS data of freeze-dried samples of cultures incubated with **137** in water indicated complete transformation of **137** in 8 h (**Figure 2.26**).
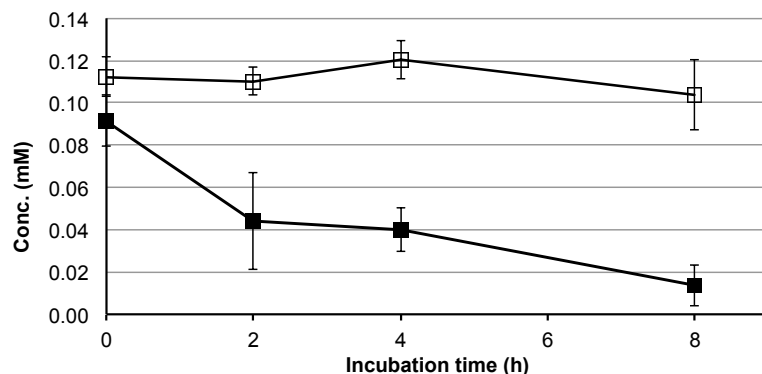
Figure 2.26 Progress curves of transformation of **137** (■) by *Alternaria brassicicola* and recovery of **137** (□) in control medium.

The HPLC-ESI-MS analysis of the 4 h samples of cultures of *A. brassicicola* incubated with **137** showed presence of 2 peaks at 12.8 and 14.6 min (Method G). The HPLC-ESI-MS (negative mode) analysis of the peak at t_R = 12.8 min indicated an ion at *m/z* 283 [M-H]⁻ which was 32 units higher than **137**, and the peak at t_R = 14.6 min at *m/z* 299 [M-H]⁻, which was 48 units higher than that of **137**. Based on these negative ions the metabolites were proposed to contain two and three additional oxygen atoms, respectively. Compounds **194** and **195** (**Figure 2.27**) were proposed to result from oxidation of compound **137**. These acids were not soluble in the EtOAc, therefore were not detected in extracts of any cultures. None of the metabolites were detected either in control cultures or in media incubated with compound **137**.

194 n = 2
195 n = 3
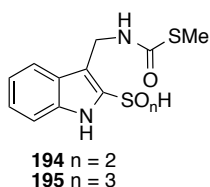
Figure 2.27   Structures of sulfinic acid **194** and sulfonic acid **195** from transformation of cyclobrassinin (**4**) by *Alternaria brassicicola*.

Similar biotransformation experiments using 1-methylcyclobrassinin (**193**) showed that it was metabolized at similar rate (ca. 8h) by *A. brassicicola* (**Figure 2.28**).
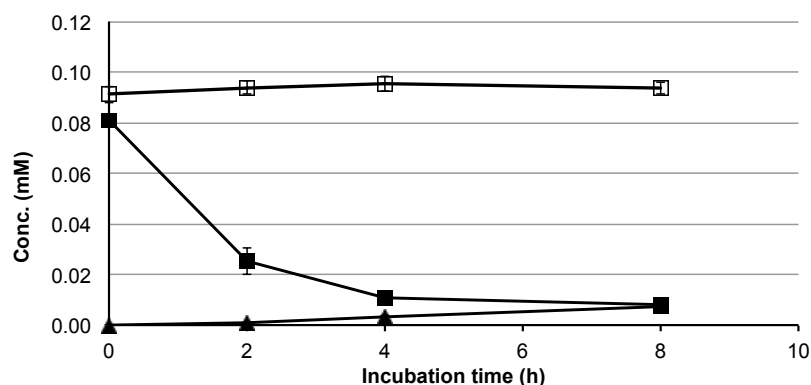
Figure 2.28 Progress curves of transformation of 1-methylcyclobrassinin (**193**, ■) by *Alternaria brassicicola*, formation of product (**196**, ▲) and recovery of methylcyclobrassinin (**193**, □) in control medium.

The chromatograms of the neutral extracts showed the presence of 1-methylcyclobrassinin (**193**, t_R = 19.3) and an additional peak at 18.8 min. ^{1}H NMR of the metabolite at 18.8 min was similar to compound **137** with an additional methyl group at δ 3.7 ppm. Considering the ^{1}H NMR data, structure **196** was proposed for the metabolite at 18.8 min (**Figure 2.29**).

196
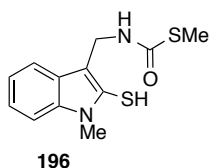
Figure 2.29 Structures of S-methyl [(1-methyl-2-sulfanyl-1*H*-indolyl-3)methyl]carbamo-thioate (**196**) from transformation of 1-methylcyclobrassinin (**193**) by *A. brassicicola*.

Compound **196** was synthesized similarly, to compound **137**, as described in **Section 3.4.2.2.1** and its data was compared with those of the isolated compound, confirming the assigned structure (Pedras et al., 2013). Compound **196** was not stable in cultures of *A. brassicicola* and was metabolized further by *A. brassicicola*. Cultures of *A. brassicicola* in water were incubated with **196** and were analyzed as described for transformation of compound **137**.

The HPLC chromatograms of the cultures of *A. brassicicola* incubated with **196** showed it transformed almost completely after 8 h (**Figure 2.31**). The HPLC-ESI-MS analysis of the 4 h samples of cultures of *A. brassicicola* incubated with **196** showed presence of 2 broad peaks at 14.5 and 15 min (method G). The HPLC-ESI-MS (negative mode) analysis of the peak at t_R = 14.5 min indicated an ion at *m/z* 297 [M-H]⁻ which was 32 units higher than **196**, and the peak at t_R = 15 min at *m/z* 313 [M-H]⁻, which was 48 units higher than that of **196**. Based on these negative ions the metabolites were proposed to contain two and three additional oxygen atoms, respectively. Compounds **197** and **198** (**Figure 2.30**) were proposed to result from oxidation of compound **196**.
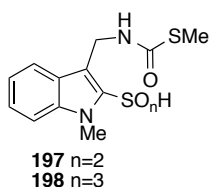
Figure 2.30   Structures of sulfinic acid **197** and sulfonic acid **198** from transformation of 1-methylcyclobrassinin (**193**) by *A. brassicicola*.
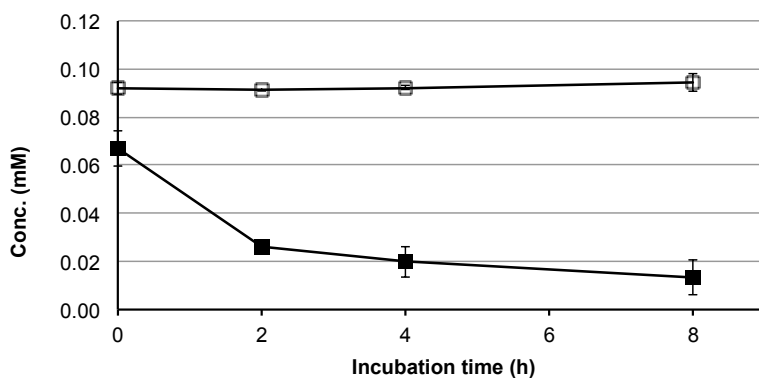
Figure 2.31   Progress curves of transformation of **196** (■) by *Alternaria brassicicola* and recovery of **196** (□) in control medium.

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2.1.2.3 Synthesis and antifungal activity of metabolites

Metabolites **137** that resulted from biotransformation of cyclobrassinin (**4**) was obtained from acid catalyzed hydrolysis of cyclobrassinin (**4**) as summarized in **Scheme 2.11** (Pedras et al., 2013). Cyclobrassinin (**4**) was hydrolyzed (HCl, 0.50 M) to metabolite **137**, the major compound, and some minor side products. Because of the poor solubility of the compound **137**, recovery of the product by extraction of the reaction mixture (neutral or acidic) was not successful (<15%). The reaction mixture was concentrated to dryness and the residue was rinsed with EtOAc or MeOH. Analysis of the soluble part in either MeOH or EtOAc did not show metabolite **137**. The residue was dissolved in DMSO-d_6 and analyzed by ^{1}H NMR, ^{13}C NMR and HMBC. The ^{1}H NMR revealed a total of 11 protons of which five were in the aromatic region (δ_H 7.0-8.5), two were for a methylene group (δ_H 3.9, *s,* 2H) and three were for methyl groups (δ_H 2.1, *s,* 3H). The ^{13}C NMR spectrum displayed signals for 11 carbons. Results of spectroscopic analyses confirmed the proposed structure for **137** (**Table 2.4**) (Pedras et al., 2013). Furthermore, the presence of tautomer **199** in DMSO-d_6 solution was ruled out from analysis of the NMR spectroscopic data.

199 R=H
200 R=Me
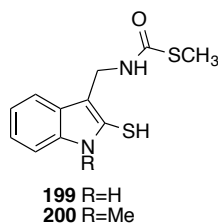
1-Methylcyclobrassinin (**193**) was subjected to similar reaction conditions as described for hydrolysis of cyclobrassinin (**4**) to yield compound **196** as the major product, together with a few side products (**Scheme 2.11**) (Pedras et al., 2013). Compound **196** was not soluble in most organic solvents; therefore DMSO-d_6 was used for spectroscopic analysis and the presence of tautomer **200** in DMSO-d_6 solution was ruled out from analysis of the NMR spectroscopic data (**Table 2.4**) (Pedras et al., 2013).
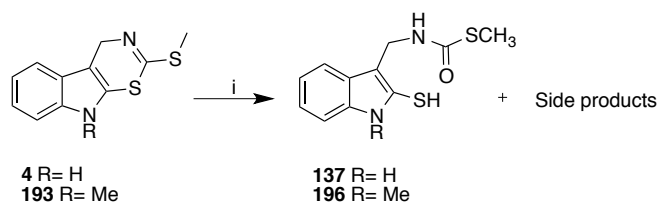
Scheme 2.11 Syntheses of **137** and **196**. Reagent and condition (i) HCl (0.5 M), 1,4-dioxane, 24 h, 40 °C, **137** (51%), **196** (35%) (Pedras et al., 2013).

Table 2.4 ^{1}H NMR and ^{13}C NMR spectroscopic data of compounds **137** (in DMSO-d_6) and **196** (in DMSO-d_6).

C/H#	δ_C 137	δ_H (m, J, H) 137	δ_C 196	δ_H (m, J, H) 196
2	126.2	-	125.2	-
3	119.7	-	120.0	-
3a	126.1	-	124.3	-
4	120.1	7.56 (d, 7.5 Hz, 1H)	115.7	7.57 (d, 7 Hz, 1H)
5	123.9	7.22 (dd, 7.5, 7.5 Hz, 1H)	123.5	7.31 (dd, 7, 8 Hz, 1H)
6	119.8	7.05 (dd, 7.5, 7.5 Hz, 1H)	119.9	7.10 (dd, 7, 8 Hz, 1H)
7	111.6	7.37 (d, 7.5 Hz, 1H)	110.5	7.53 (d, 8 Hz, 1H)
7a	137.4	-	138.1	-
1'	34.4	3.92 (brd, 2.5 Hz, 2H)	34.5	3.64 (br, 2H)
2'	165.8	-	165.6	-
(N-1)H	-	11.57 (brs, 1H)	-	-
(N)H	-	8.22 (brt, 2.5 Hz, 1H)	-	8.20 (brt, 1H)
(S)CH$_3$	11.5	2.12 (s, 3H)	11.4	2.10 (s, 3H)
(N)CH$_3$	-	-	29.8	3.67 (s, 3H)

To determine the structure of the side products resulting from hydrolysis of cyclobrassinin (**4**) (**Scheme 2.12**), the EtOAc extract of the reaction mixture was analyzed by

HPLC-DAD. The HPLC chromatograms of the extract displayed two peaks at 17.9 and 26.0 min, which were separated by FCC and characterized. The compound at 17.9 min (<5%) displayed a molecular formula of $C_{18}H_{14}N_2S_2$, as suggested by HRMS-EI. The ^1H NMR (CDCl$_3$) spectrum of the metabolite at 17.9 min displayed signals for 14 protons: eight aromatic (δ_H 7.0-7.5), two D$_2$O exchangeable (δ_H 9.51 and 7.94) and four methylenic protons (δ_H 3.65, d, J=16Hz, 1H; 2.83, dd, J=16, 2Hz, 1H; 3.87, d, J = 13 Hz, 1H; 2.64, dd, J = 13, 2 Hz, 1H). The ^{13}C NMR spectrum displayed signals for 18 carbons (**Table 2.6**). Analysis of the HMBC and HMQC indicated that the proton at δ_H 3.65 correlated to the C=S at δ_c 210.8 and that methylene protons at δ_H 3.65 and 2.83 correlated with a quaternary carbon at δ_C 106.2. Based on these results, structure **201** was suggested for the compound with t_R = 17.9 min (**Scheme 2.12**) (Pedras et al., 2013). Another product obtained together with **201** could not be purified completely.

201
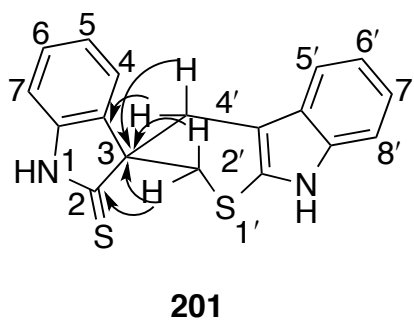
 Figure 2.32   Selected HMBC correlation of compound **201**.

Similarly, hydrolysis of 1-methylcyclobrassinin (**193**) gave some minor products, of which two (t_R = 26.0 and 24.8 min) were purified by preparative TLC (<10%) and spectroscopically characterized (Pedras et al., 2013). The purified compounds were structural isomers, as suggested by their identical molecular formulas ($C_{20}H_{18}N_2S_2$) obtained from HRMS (EI and ESI) and NMR spectral data. Similar to compound **201**, obtained from hydrolysis of cyclobrassinin (**4**), the ^1H NMR spectrum of the compound at 26.0 min displayed two independent aromatic spin systems of four protons each, two spin systems of methylene protons (δ_H 3.88, d, J = 13 Hz, 1H; 2.58, dd, J = 13, 2 Hz, 1H; 3.64, d, J = 16 Hz, 1H; 2.77, dd, J = 16, 2Hz, 1H) and two methyl singlets. The ^{13}C NMR spectrum displayed signals for 20 carbons, of

which a carbon signal at δ_C 208.6 was assigned to a thiocarbonyl group. This spectroscopic analysis suggested structure **203** for one of the isomers (Pedras et al., 2013). The ^{1}H NMR of the minor compound (t_R = 24.8 min) displayed two independent aromatic spin systems of four protons each, and a spin systems of two methylene protons, which were different from those of isomer **203** (δ_H 3.33, ddd, J = 17, 12, 6 Hz and 3.10, ddd, J = 17, 12, 6Hz; 2.85, ddd, J = 14, 12, 6 Hz and 2.04, ddd, J = 14, 12, 6 Hz) (**Table 2.6**). The coupling constants showed that the methylene protons of this isomer were adjacent to each other. These spectroscopic results suggested structure **202**, a structural isomer of **203**, for this compound. Detailed analysis of the HMBC and HSQC of each isomer was consistent with these proposed structures. For example the methylene protons of **202** at δ_H 3.33 correlated with carbon at δ_C 104.9 and a quaternary sp^3 carbon at δ_C 62.5, as summarized in **Figure 2.33**.

202
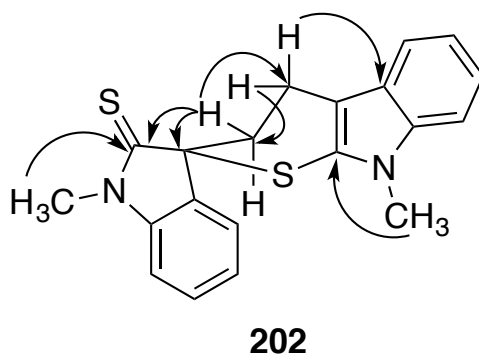
Figure 2.33   Selected HMBC correlation of compound **202**.

4 R= H
193 R= Me

137 R= H
196 R= Me

201 R= H
203 R= Me

204 R= H
202 R= Me
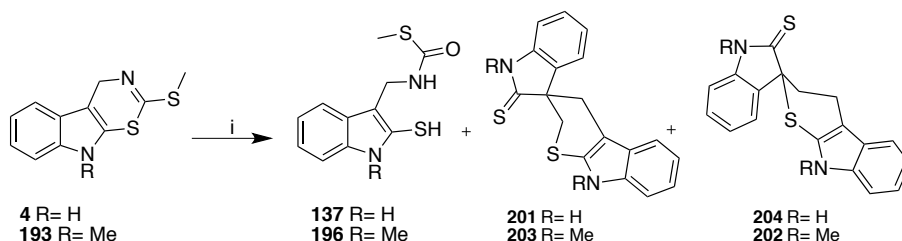
Scheme 2.12 Hydrolysis of cyclobrassinin (**4**) and 1-methylcyclobrassinin (**193**). Reagent and condition: (i) HCl (0.50 M), 1,4-dioxane, 24 h, 40 °C.

Importantly, while structures **201-204** could be justified as products of [4+2] cycloadditions, that is, Diels-Alder reactions of 3-methyleneindoline-2-thiones **205** and **206**, the formation of **202** and **204** appeared to be unique. For this reason, spirocyclic indoline-2-thiones were prepared using another procedure, as follows.

205 R= H
206 R= Me
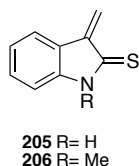
Figure 2.34   Structures of 3-methyleneindoline-2-thiones **205** and **206**.

209   **210**
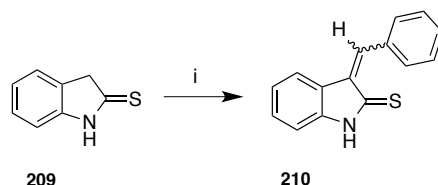
Scheme 2.13 Synthesis of (*E* and *Z*)-3-benzylideneindoline-2-thione (**210**). Reagents and condition: (i) PhCHO, conc. HCl/EtOH, 20 °C (Thompson et al., 1993).

Condensation of 1-methylindoline-2-thione (**207**) with formaldehyde under acidic condition followed by [4+2] intermolecular cycloaddition yielded a mixture of compounds **203** and **202** in a 1:1 ratio (32% yield) (**Scheme 2.14**) were separated using FCC for further analyses (**Table 2.6**) (Pedras et al., 2013).

207 R= CH$_3$ **203** R= CH$_3$ **202** R= CH$_3$
208 R= OMe   **211** R= OMe   **212** R= OMe
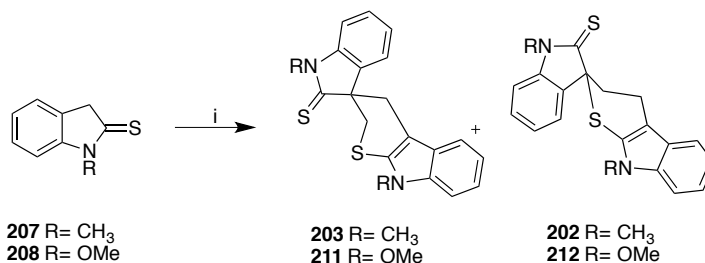
Scheme 2.14 Syntheses of spirocyclic indoline-2-thiones **203**, **202**, **211** and **212**. Reagents and condition: (i) HCHO, MeOH, HCl, r.t (**Table 2.5**).

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Similarly, condensation of 1-methoxyindoline-2-thione (**208**) with formaldehyde proceeded under acid catalysis and was followed by cycloaddition to afford compounds **211** and **212** in a 1.2:1 ratio (46% yield) (**Scheme 2.14**)(**Table 2.6**) (Pedras et al., 2013). Several attempts to obtain compounds **201** and **204** from condensation of indole-2-thione (**209**) with formaldehyde yielded multiple products, of which **201** was a minor component of the reaction mixture as determined by the HPLC analysis. Spiroindolinethiones **202**, **203**, **211** and **212** were prepared using either HCl or TFA, as summarized in **Table 2.5**, but were obtained in better yield using HCl (Pedras et al., 2013).

Table 2.5 Synthesis of spiroindolinethiones **203**, **202**, **211** and **212** and reaction conditions.

Indoline-2-thiones	Conditions	Products (ratio)	Yield (%)
207	HCl	**203**+**202** (1:1)	32
208	HCl	**211**+**212** (1.2:1)	46
207	TFA	**203**+**202** (1:1)	20
208	TFA	**211**+**212** (1.2:1)	33

As it was described, the formation of **202** or **212** from [4+2] intermolecular cycloaddition of 3-methyleneindoline-2-thiones **205**, **206** and **215** is unprecedented. Therefore, to further confirm the structures crystallization of **212** in hexane-Et$_2$O solution yielded crystals suitable for X-ray crystallography as shown in **Figure 2.35**. This analysis confirmed the structural assignment based on NMR spectroscopic data (Pedras et al., 2013).
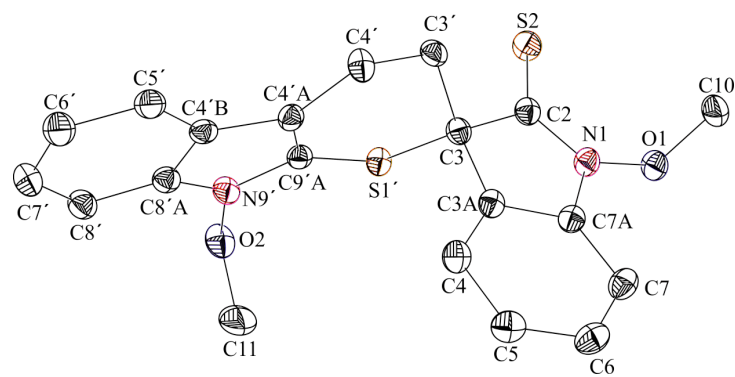
Figure 2.35 Molecular structure of compound **212** with thermal ellipsoids at 30% of the probability level (hydrogen atoms omitted for clarity).

Table 2.6 ^{1}H NMR and ^{13}C NMR spectroscopic data of compounds **201, 203, 202, 211** and **212** (in CDCl$_3$).

C/H #	δ$_C$ 202	δ$_H$ (m, J, H) 202	δ$_C$ 203	δ$_H$ (m, J, H) 203	δ$_C$ 201	δ$_H$ (m, J, H) 201	δ$_C$ 211	δ$_H$ (m, J, H) 211	δ$_C$ 212	δ$_H$ (m, J, H) 212
2	203.2	-	208.6	-	210.8	-	198.4	-	193.7	-
3	62.5	-	54.0	-	54.4	-	52.5	-	60.3	-
3a	136.5	-	135.5	-	135.7	-	133.9	-	134.2	-
4	124.2	7.11 (d, 7.5 Hz, 1H)	126.4	7.21(d, 7.5 Hz, 1H)	126.7	7.23 (d, 7.5 Hz, 1H)	126.6	7.43 (d, 10 Hz, 1H)	117.3	7.4 (d, 10 Hz, 1H)
5	124.9	7.01 (dd, 7.5, 7.5 Hz, 1H)	124.1	7.03 (dd, 7.5, 7.5 Hz, 1H)	123.9	7.01 (ddd, 7.5, 7.5, 1 Hz, 1H)	120.3	7.22 (m, 3H)	120.4	7.1 (m, 2H)
6	129.1	7.36 (dd, 7.5, 7.5 Hz, 1H)	128.4	7.36 (dd, 7.5, 7.5 Hz, 1H)	128.5	7.31 (ddd, 7.5, 7.5, 1 Hz, 1H)	128.7	7.38 (dd, 10, 5 Hz, 1H)	129.4	7.30 (dd, 10, 5 Hz, 1H)
7	109.7	7.09(d, 7.5 Hz, 1H)	109.3	7.10 (d, 7.5 Hz, 1H)	109.7	7.08 (d, 7.5 Hz, 1H)	108.2	7.29 (d, 10 Hz, 1H)	108.2	7.33 (d, 10 Hz, 1H)
7a	145.5	-	144.2	-	141.3	-	140.3	-	139.7	-
3′	18.5	3.33 (ddd, 17, 12, 6 Hz, 1H) 3.10 (ddd, 17, 12, 6 Hz, 1H)	32.9	3.64 (d, 16 Hz, 1H) 2.77 (dd, 16, 2 Hz, 1H)	32.4	3.65 (d, 16 Hz, 1H) 2.83 (dd, 16, 2 Hz, 1H)	31.9	3.86 (d, 15 Hz, 1H) 2.73 (d, 15 Hz, 1H)	18.2	3.33 (dm, 1H) 3.04 (m, 1H)
4′	34.8	2.85 (ddd, 14, 12, 6 Hz, 1H) 2.04, (ddd, 14, 12, 6 Hz, 1H)	37.1	3.88, 1H, d, 13Hz 2.58 (dd, 13, 2 Hz, 1H)	37.1	3.87, 1H, d, 13Hz 2.64 (dd, 13, 2 Hz, 1H)	36.3	3.58 (d, 13 Hz, 1H) 2.63 (d, 10 Hz, 1H)	34.4	2.79 (m, 1H) 2.10, (dm, 1H)
Others	29.8	3.59 (s, 3H)	32.0	3.78 (s, 3H)	-	7.94 (s, N-H)	65	4.2 (s, 3H)	65.1	4.12 (s, 3H)
9'a	127.6	-	127.5	-	124.7	-	124.9	-	124.2	-
4'a	104.9	-	104.8	-	106.2	-	102.7	-	103.1	-
4′b	127.4	-	128.1	-	128.5	-	128.7	-	125.6	-

C/H #	δ_C 202	δ_H (m, J, H) 202	δ_C 203	δ_H (m, J, H) 203	δ_C 201	δ_H (m, J, H) 201	δ_C 211	δ_H (m, J, H) 211	δ_C 212	δ_H (m, J, H) 212
5'	117.0	7.51 (d, 7.5 Hz, 1H)	116.7	7.32 (d, 7.5 Hz, 1H)	116.7	7.34 (d, 7.5 Hz, 1H)	116.9	7.22 (m, 3H)	121.8	7.16 (d, 5 Hz, 1H)
6'	119.3	7.16 (dd, 7.5, 7.5 Hz, 1H)	119.2	7.06 (dd, 7.5, 7.5 Hz, 1H)	119.8	7.10 (dd, 7.5, 7.5 Hz, 1H)	120.3	7.08 (ddd, 10, 5, 5 Hz, 2H)	125.5	6.99 (dd, 10, 5 Hz, 1H)
7'	120.8	7.21, (dd, 7.5, 7.5 Hz, 1H)	120.8	7.18 (dd, 7.5, 7.5 Hz, 1H)	121.5	7.18 (dd, 7.5, 7.5 Hz, 1H)	121.8		120.4	7.1 (m, 2H)
8'	108.4	7.30 (d, 7.5 Hz, 1H)	108.2	7.31 (d, 7.5 Hz, 1H)	110.1	7.36 (d, 7.5 Hz, 1H)	107.2	7.22 (m, 3H)	108.5	7.03 (d, 5 Hz, 2H)
8'a	137.8	-	137.5		136.5	-	132.4	-	134.2	-
Others	32.1	3.74 (s, 3H)	30.1	3.77 (s, 3H)	9.51 (s, N-H)-	9.51 (s, N-H)-	62.2	4.1 (s, 3H)	62.2	3.88 (s, 3H)

Compounds **137** and **196** were further characterized after methylation of the thiol group using MeI (**Scheme 2.15**). The reaction was carried out using NaH. Compound **213** was obtained 5 min after addition of NaH; longer reaction times (45 min) yielded compound **214** (**Scheme 2.15**).
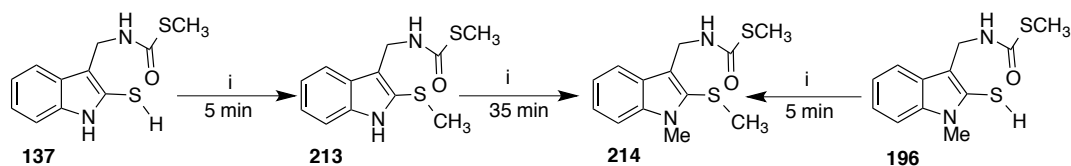
Scheme 2.15 Syntheses of **213** and **214**. Reagents and conditions (i) NaH, MeI, THF, 0°C.

The chemical synthesis of compounds **194** and **195** were carried out to confirm the chemical structures of the final biotransformation products of cyclobrassinin (**4**) and to obtain sufficient amounts for determination of their antifungal activities. Preparation of compounds **194** and **195** was attempted by oxidation of **137** using *m*-CPBA or H_2O_2. Compound **194** was obtained by oxidation of compound **137** with H_2O_2 in good yield (**Scheme 2.16**).
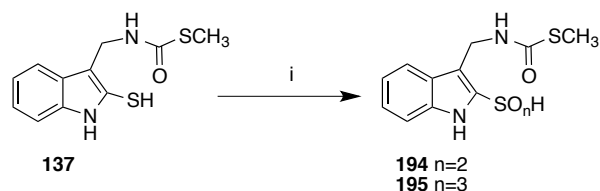
Scheme 2.16 Syntheses of sulfinic acids **194** and **195**. Reagent and conditions (i) MeOH/CHCl₃, (1:1), r.t., H_2O_2 (16.5eq),16 h, **194** (91%) or H_2O_2 (32 eq), 20 h, **195**.

Due to the low solubility of the product in organic solvents, the reaction mixture was concentrated to dryness and the residue was rinsed with MeOH. Most of the residue remained insoluble in MeOH. ^{1}H NMR (DMSO-*d₆*) spectrum of the residue displayed signals for 11 protons, of which six were aromatic (δ_H 7.1-8.2), two were from a methylene group (δ_H 3.9, *s, 2H*) and three were from a methyl group (δ_H 2.1, 3H). The HPLC-ESI-MS analysis of the sample

showed a peak at 12.8 min containing an ion (negative mode): [M-1]⁻ at *m/z* 283. This structural analysis suggested structure **194**. Increasing the time of the reaction as well as using excess amount of H_2O_2 gave sulfonic acid **195** as the major compound. ^{1}H NMR of the crude reaction mixture in DMSO-*d₆* was similar to that obtained for acid **194**. The HPLC-ESI-MS analysis of the crude reaction mixture showed a broad peak at 8 min containing an ion (negative mode) at *m/z* 299 [M-1]⁻. These results suggested structure **195**. HPLC-ESI-MS chromatogram of the crude also showed acid **194** as minor product in the crude reaction mixture.

Another method to prepare acids **194** and **195** was attempted by oxidation of compound **137** with *m*-CPBA; however this condition lead to mixture of both acids **194** and **195**.
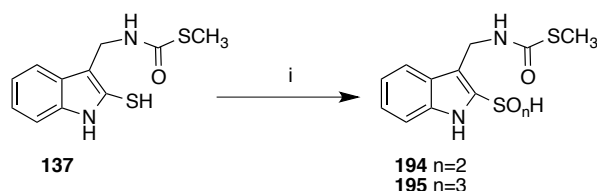
Scheme 2.17 Syntheses of sulfinic acid **194** and sulfonic acid **195**. Reagent and condition (i) *m*-CPBA, MeOH, -20 °C.

In summary, conditions to obtain sulfonic acid **195** as a single reaction product were used namely various time and temperature, however mixture of acids **194** and **195** was obtained in all conditions. The mixture could not be separated by chromatography.

To establish if biotransformations of cyclobrassinin (**4**) and 1-methylcyclobrassinin (**193**) by *A. brassicicola* were detoxification reactions, mycelial radial growth assays were carried out. The antifungal activities against *A. brassicicola* of all metabolites and the spiro compounds **203**, **202**, **211** and **212** were determined as described in **Section 3.2**; results of these assays are shown in **Table 2.7**.

Table 2.7 Antifungal activity of metabolites **137, 39** and spiro compounds **203**, **202**, **211** and **212** against *Alternaria brassicicola*.

Compound (#)	Inhibition ± SD (%)[a]		
	0.50 mM	0.20 mM	0.10 mM
Hyd-cyclobrassinin (**137**)	$14 \pm 3^{e,f}$	n. i.	n. i.
Hyd-methylcyclobrassinin (**196**)	24 ± 0^{c}	$20 \pm 3^{d,e}$	$15 \pm 3^{d,e,f}$
Methylspiro (**203**)	$27 \pm 3^{b,c}$	$22 \pm 3^{c,d}$	$15 \pm 0^{d,e,f}$
Methylspiro (**202**)	25 ± 0^{c}	$22 \pm 3^{c,d}$	$13 \pm 3^{e,f}$
Methoxyspiro (**211**)	33 ± 3^{b}	23 ± 3^{c}	$13 \pm 3^{e,f}$
Methoxyspiro (**212**)	25 ± 2^{c}	$13 \pm 3^{e,f}$	8 ± 3^{f}

[a]The percentage of inhibition was calculated using the formula: % inhibition = 100 − [(growth on amended/growth in control) × 100]; values are averages of three independent experiments conducted in triplicate; n.i. = no inhibition. For statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted α set at 0.05; n = 3; different letters in the same column ([b]–[g]) indicate significant differences ($P < 0.05$).

Metabolite **137** from transformation of cyclobrassinin (**4**) showed low antifungal activity against *A. brassicicola*. Compound **137** showed 14% inhibitory activity against mycelial growth of *A. brassicicola* only at highest tested concentration (0.50 mM). Hydrolyzed product **196** from transformation of 1-methylcyclobrassinin (**193**) showed slightly stronger inhibitory activity than metabolite **137** against *A. brassicicola*. At highest concentration (0.50 mM), compound **196** showed 24% mycelial growth inhibition. The activities of spirocompounds **202**, **203**, **211** and **212** were also determined against *A. brassicicola*. All tested spirocompounds showed similar antifungal activity against *A. brassicicola* (**Table 2.7**).

2.1.2.4 Discussion and conclusion

Cyclobrassinin (**4**) and 1-methylbrassilexin (**193**) displayed weak antifungal activity against mycelial growth of *A. brassicicola* compared to camalexin (**1**, 100% at 0.20 mM) or brassilexin (**7**, 100% at 0.50 mM). At the highest concentration (0.50 mM) 1-methylcyclobrassinin (**193**) showed stronger inhibitory activity than cyclobrassinin (**4**) against mycelial growth of *A. brassicicola*. However, both compounds showed very similar activity against *A. brassicicola* at lower (0.20 and 0.10 mM) concentrations. The spiro compounds showed antifungal activity against *A. brassicicola* that was around 30% for the highest concentration (0.50 mM).

Cyclobrassinin (**4**) and 1-methylcyclobrassinin (**193**) were metabolized completely after 8 h by *A. brassicicola* to compounds **137** and **196**, at similar rates. Compounds **137** and **196** were transformed further by *A. brassicicola* to the corresponding acids **194**, **197**, **195** and **198** (**Scheme 2.18**). It is noteworthy that detoxification of cyclobrassinin (**4**) is rather fast, unlike the metabolism of the phytoalexin camalexin (**1**) (ca. 10 days at 0.50 mM) by *A. brassicicola* (Pedras and Abdoli, 2013). The pathway of transformation of cyclobrassinins by *A. brassicicola* is proposed in **Scheme 2.18**.
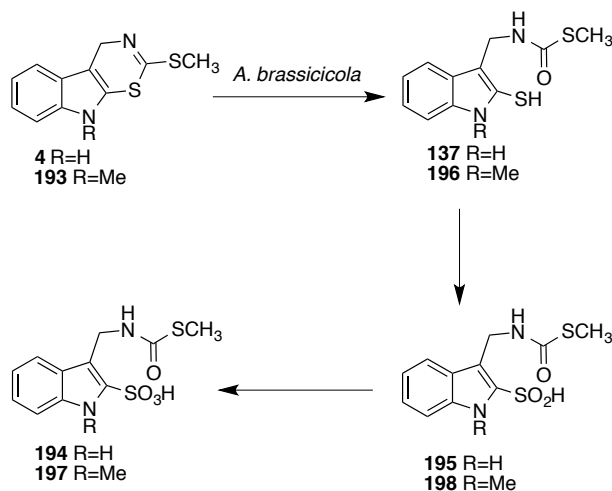
Scheme 2.18 Proposed pathway of detoxification of cyclobrassinin (**4**) and 1-methylcyclobrassinin (**193**) by *Alternaria brassicicola* and metabolic products.

Comparison of the antifungal activities (**Table 2.3** and **Table 2.7**) suggested that growth inhibition of the metabolites was weaker than that caused by the parent compounds. The antifungal activity of acids **194**, **195**, **197** and **198** were not determined against *A. brassicicola*. Results of antifungal activity of these acids are necessary to confirm that biotransformations of cyclobrassinin (**4**) and 1-methylcyclobrassinin (**193**) are detoxifications.
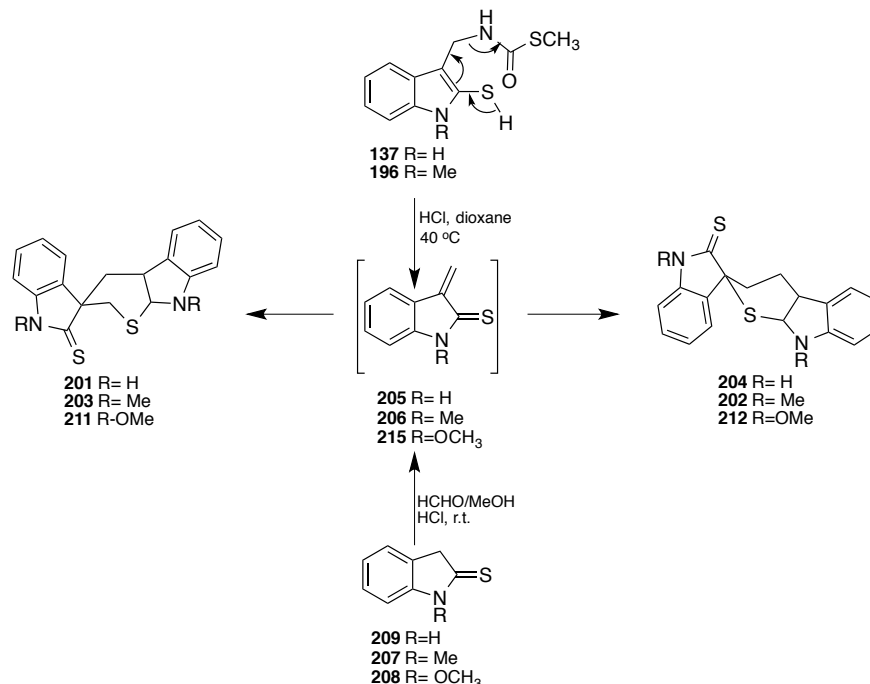
Scheme 2.19 Chemical transformations of compounds **137**, **196**, **207** and **208** to spirocyclic indoline-2-thiones **201**, **204**, **203**, **202**, **211** and **212**.

As described above, compounds **201, 202, 203**, **204**, **211** and **212** were obtained from [4+2] cycloaddition of corresponding 3-methyleneindoline-2-thiones (**Scheme 2.19**) (Pedras et al., 2013). The formation of compounds **202** or **212** resulting from [4+2] intermolecular cycloadditions of 3-methyleneindoline-2-thiones **207** or **208** is unprecedented. By contrast, the formation of **203** and **211** from [4+2] intermolecular cycloadditions has literature precedent (Thompson et al., 1993). It was surprising to find that both isomers from each reaction were produced in the same amount. In addition, it was established that purified products **211** and **212** are stable under conditions identical to those used in the condensation reactions and did not

interconvert (Pedras et al., 2013).

In conclusion, it was shown for the first time that the plant pathogen *A. brassicicola* was able to detoxify cyclobrassinin (**4**) and 1-methylcyclobrassinin (**193**) in 8 h, using a transformation pathway, that involved hydrolyses to compounds **137** and **196**. Compounds **137** and **196** were metabolized further by *A. brassicicola* to their corresponding sulfinic and sulfonic acids **194** and **197**. Previously it had been shown that cyclobrassinin (**4**) can inhibit the fungal enzyme brassinin hydrolase (BHAb) from *Alternaria brassicicola* (Pedras et al., 2012), which catalyzes brassinin (**9**) detoxification to indole-3-methanamine (**28**). However, these results suggest that cyclobrassinin (**4**) is not of interest as a lead structure to design paldoxins against *A. brassicicola* because it is quickly metabolized by this plant pathogen (Pedras et al., 2013).

2.1.3   Rutalexin

Rutalexin (**5**) was first isolated from stressed (UV light) rutabaga tubers (*Brassica napus* L. ssp. *rapifera*) and its synthesis confirmed the proposed structure (Pedras et al., 2004b). Due to poor solubility of rutalexin (**5**) in agar, its antifungal activity was determined against *Cladosporium cucumerinum* using a TLC bioassay that showed complete inhibition at 2×10^{-6} mol (Pedras et al., 2004b).
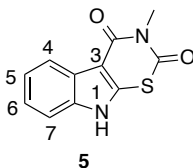
Figure 2.36   Structure of rutalexin (**5**).

2.1.3.1   Synthesis and antifungal activity

In a previously reported procedure (Pedras et al., 2004b), rutalexin (**5**) was synthesized in 24% overall yield from *N*-Boc-2-chloroindole-3-carboxaldehyde (**15**), via sulfanylamide **18**. Because of the low yield (24% overall) of **5** in the reported procedure, the first part of this work was dedicated to optimize rutalexin (**5**) synthesis. Since sulfanylamide **18** was prepared in good yield (Pedras et al., 2004b), it was used in this work as an intermediate. However, it was discovered that sulfanylamide **18** was not stable; upon acidic work-up it oxidized spontaneously to disulfide **219**. The structure of sulfanylamide **18** was confirmed by methylation of its thiol group followed by deprotection using TFA (20%) in DCM to afford **218** in 91% overall yield (**Scheme 2.20**). To prevent the oxidation of **18**, methyl chloroformate was added directly to the reaction mixture containing sulfanylamide **18,** which after 30 min at room temperature afforded compound **216** in almost quantitative yield (95%). Cyclization of amide **216** in the presence of Et$_3$N followed by deprotection using TFA yielded crude rutalexin (**5**). The final reaction mixture was concentrated to dryness and the residue was rinsed with Et$_2$O to yield pure rutalexin (**5**) in 93% yield over two steps (**vi** and **vii**) and about 80% overall yield (from the aldehyde **15**) (**Scheme 2.20**). In another attempt to simplify the described procedure, amide **216** was allowed

to react for longer time to cyclize and yield *N*-Boc-rutalexin **19**. Compound **19** was separated from the crude reaction mixture using FCC in 56% yield (from amide **17**, **Scheme 2.20**). Although this is a simple procedure, the yield was not as good as in the previous transformation. Alternatively, *N*-Boc deprotection of compound **19** using K_2CO_3 afforded rutalexin (**5**) in 60% yield (step **viii**).
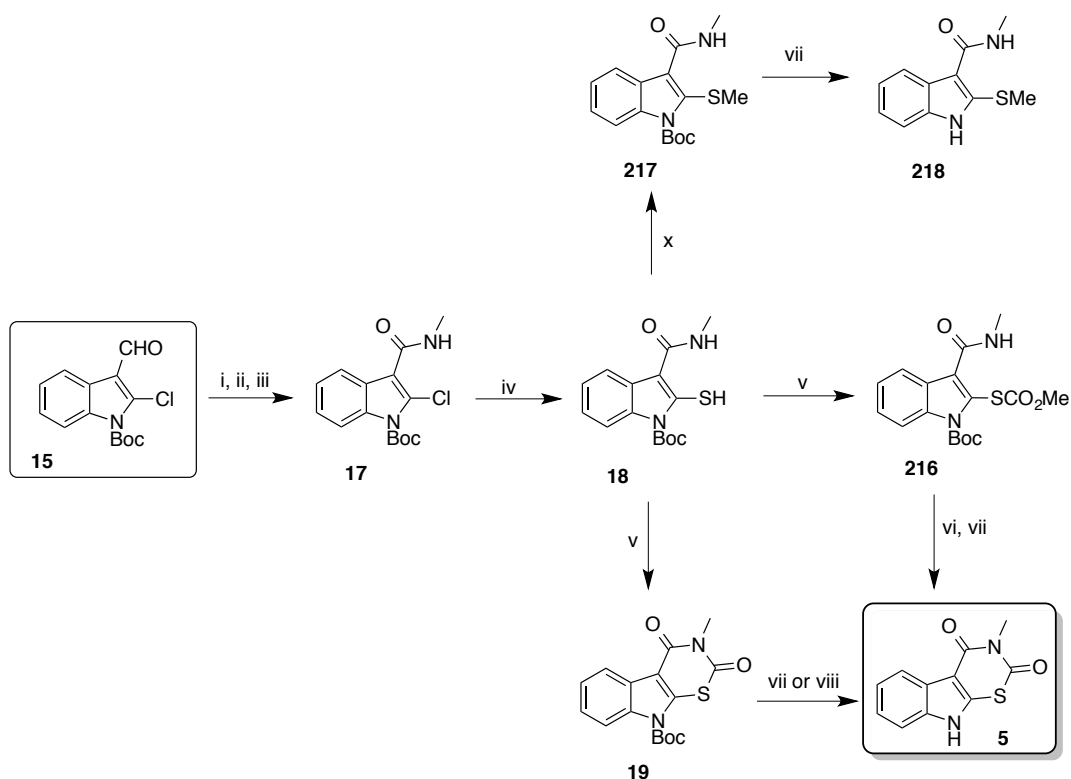
Scheme 2.20 Syntheses of rutalexin (**5**) and **218**. Reagents and conditions: (i) $NaClO_2$, KH_2PO_4, 2-methylbut-2-ene, *t*-butanol, H_2O, r.t., 100%; (ii) $SOCl_2$, DMF, THF, r.t.; iii) CH_3NH_2, THF, 0 °C, 95% (over steps **ii** and **iii**); (iv) NaSH, DMF, H_2O, 0 °C, 1 h; (v) Methyl chloroformate, 30 min, **216** (95%) and 6 h, **19** (56%); (vi) THF, Et_3N, r.t., 4 h; (vii) TFA (20%) in DCM, r.t., 4 h, 93% (over 2 steps); (viii) K_2CO_3, MeOH, r.t., 2 h, 60%; (x) MeI, THF, r.t., 30 min, 97%.

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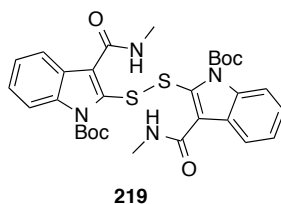
Figure 2.37   Structure of compound **219**.

Due to low solubility in agar medium, the antifungal activity of rutalexin (**5**) was determined against *A. brassicicola* employing a mycelial radial growth assay in liquid minimal medium (not in solid agar medium), as described in the experimental **Section 3.2**. The antifungal activity was determined using two different concentrations (0.10 and 0.05 mM). Results of these assays are shown in **Table 2.8**.

Table 2.8   Antifungal   activity   of   rutalexin   (**5**)   against *Alternaria brassicicola.*

Compound (#)	Inhibition ± SD (%)[a]	
	0.10 mM	0.050 mM
Rutalexin (**5**)	20 ± 4[d]	12 ± 3[e]
Camalexin (**1**)	76 ± 0[b]	25 ± 5[c]

[a]The percentage of inhibition was calculated using the formula: % inhibition = 100 − [(growth on amended/growth in control) × 100]; values are averages of three independent experiments conducted in triplicate. For statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted α set at 0.05; n = 3; different letters ([b]–[e]) indicate significant differences (P < 0.05).

Results of the antifungal activity assays indicated that the inhibitory activity of rutalexin (**5**) against *A. brassicicola* (**Table 2.8**) is not as strong as camalexin (**1**) (76%, 0.10 mM, **Table 2.1**). At the highest concentration (0.10 mM) rutalexin (**5**) showed only 20% inhibitory against mycelial growth of *A. brassicicola*, which decreased to 12% at 0.05 mM (**Table 2.8**).

2.1.3.2   Biotransformation of rutalexin

The transformation of rutalexin (**5**) by *A. brassicicola* was investigated. Due to the moderate inhibitory activity of rutalexin (**5**) against *A. brassicicola*, the metabolic studies were carried out using 0.10 mM of rutalexin (**5**). Cultures of *A. brassicicola* were incubated for different periods of time and samples were collected from each culture immediately after addition of rutalexin (**5**) up to several days. Samples of cultures were extracted and analyzed by HPLC-DAD-ESI-MS. Control solutions containing rutalexin (**5**) (MM only, no fungus) were analyzed similarly to determine the chemical stability of rutalexin (**5**) during the incubation experiment.
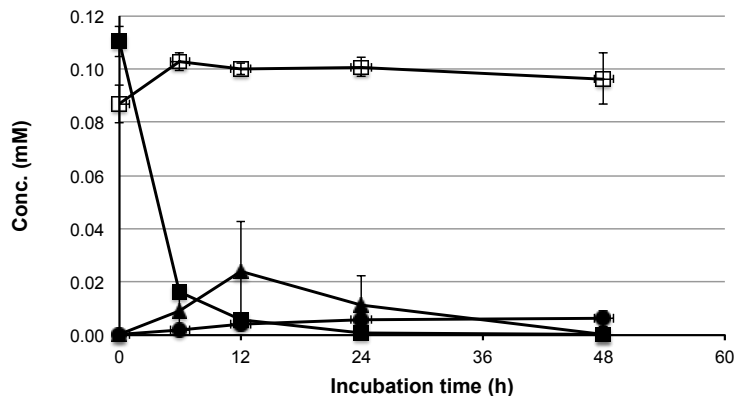
Figure 2.38   Progress curves of transformation of rutalexin (**5**, ■) by *Alternaria brassicicola* and formation of disulfide **220** (▲) and **221** (●) and recovery of rutalexin (**5**, □) in control medium.

The HPLC chromatograms of the neutral extracts of cultures of *A. brassicicola* incubated with rutalexin (**5**) showed that it was transformed in ca. 24 h (**Figure 2.38**). The chromatograms of the neutral extracts obtained after 12 h of incubation displayed the corresponding peak of rutalexin (**5**) at 8.4 min and additional peaks at 9.9 and 10 min. The peaks at 9.9 and 10 min (HPLC method C) were not detected in either fungal cultures without rutalexin (**5**) or MM incubated with rutalexin (**5**). No additional peaks were detected either in acidic/basic extracts or in controls. The HPLC-ESI-MS (negative mode) analysis of the peak at t_R = 10 min indicated an ion at *m/z* 409 [M-H]⁻. Based on this negative ion metabolite was proposed to be disulfide **220**

93

(**Figure 2.39**), which had resulted from oxidation of sulfanylamide **223** (**Scheme 2.23**). This structure was confirmed by direct comparison with an authentic synthetic sample.

220
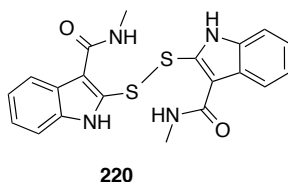
Figure 2.39   Structure of disulfide **220** (t_R = 10 min) from transformation of rutalexin (**5**) by *Alternaria brassicicola.*

Larger scale mycelial cultures incubated with rutalexin (**5**) were extracted, the extract was fractionated by reverse phase silica gel chromatography and each fraction was analyzed by HPLC. The metabolite at 9.9 min displayed a molecular formula of $C_{24}H_{28}N_2O_5S$, suggested by HRMS-EI and consistent with the NMR spectroscopic data. The ^1H NMR spectrum (CD_3CN) displayed signals for 28 protons: five aromatic (δ_H 7–7.9 ppm), two D_2O exchangeable (δ_H 7.3–9.9 ppm), two vinyl (δ_H 6 and 6.3 ppm), one methyl attached to oxygen (δ_H 3.8 ppm), one methyl attached to –NH (2.9 ppm), and four additional methyl groups. The ^{13}C NMR spectrum displayed signals for 24 carbons **Table 2.9**. These NMR data indicated the presence of an indolyl moiety containing an amide at C-3 and sulfur at C-2. Detailed analysis of the HSQC and HMBC data indicated that the proton at δ_H 6.37 correlated to C_6, C_9 and C_{12} at δ_C 160.5, 76.8 and 26.8, respectively; δ_H 6.10 correlated to C_3, C_6 and C_7 at δ_C 102.5, 160.5 and 129.7, respectively; the methyl protons at δ_H 1.40 correlated with C_9 at 76.8. NOE data showed correlations between H_5 and H_{13}, H_{13} and H_8 (**Figure 2.40**). Comparison of ^1H and ^{13}C NMR spectroscopic data of the unknown metabolite with ^1H and ^{13}C NMR data of phomapyrone G, a secondary metabolite reported from *A. brassicicola* (Pedras et al., 2009a), suggested it to be part of the unknown metabolite. Namely, the presence of two characteristic singlets at 6.1 and 6.3 ppm in ^1H NMRs of the unknown metabolite (at 9.9 min) and phomapyrone G (**75**).
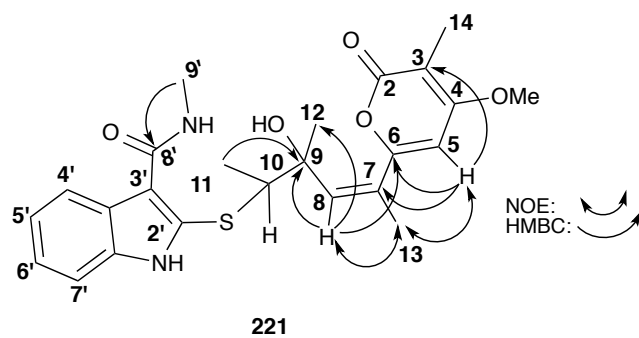
Figure 2.40 Structure of adduct **221** (t_R = 9.9 min) from transformation of rutalexin (**5**) by *Alternaria brassicicola*.

Table 2.9 ^{1}H NMR and ^{13}C NMR spectroscopic data of adduct **221** (in CD$_3$CN).

C/H#	δ$_C$ **221**	δ$_H$ (m, J, H) **221**
2	165.2	-
3	102.5	-
4	167.1	-
5	94	6.1 (s, 1H)
6	160.5	-
7	129.7	-
8	136.8	6.4 (s, 1H)
9	76.8	-
10	57.6	3.5 (q, J= 5Hz, 1H)
11	17.6	1.4 (d, J = 7Hz 3H)
12	26.8	1.5 (s, 3H)
13	13.5	2.1 (s, 3H)
14	9	1.8 (s, 3H)
2′	130.9	-
3′	115.63	-
3′a	127.8	-
4′	111.9	7.30 (d, J =8Hz, 1H)
5′	124.1	7.07 (dd, J =7.5, 7.5 Hz 1H)
6′	121.7	7.14 (dd, J =7, 7Hz, 1H)
7′	121.8	7.95 (d, J =8Hz, 1H)
7′a	137.3	-
8′	166.4	-
OH	-	4.2 (b, 1H)
(N-1)H	-	9.90 (b, 1H)
(NH)	-	7.33 (b, 1H)
(O)CH$_3$	57.3	2.12 (s, 3H)
(NH)CH$_3$	26.4	2.9 (d, J = 5Hz, 3H)

Next, disulfide **220** was administrated to cultures of *A. brassicicola*. The cultures were incubated, extracted and analyzed by HPLC-DAD-ESI-MS, as described above for rutalexin (**5**). Time-course studies revealed that disulfide **220** was metabolized within 24 h by *A. brassicicola*,

but no metabolites could be detected in the extracts of the cultures incubated with disulfide **220**. Cultures of *A. brassicicola* were prepared in water as described in **Section 3.4.3.1** and were incubated with disulfide **220**. Samples were collected at various times, freeze-dried, the residue was dissolved in MeOH-H$_2$O (50:50) and analyzed directly by HPLC-DAD-ESI-MS. HPLC-ESI-MS data of freeze-dried samples showed presence of a broad peak around 15 min (Method G). The HPLC-ESI-MS (negative mode) analysis of this peak indicated an ion at *m/z* 253 [M-H]$^-$, which was 48 units higher than that of sulfanylamide **220**. Based on this negative ion, metabolite was proposed to contain three additional oxygen atoms. Metabolite was proposed to be sulfonic acid **222**. The structure of acid **222** was confirmed by direct comparison with an authentic synthetic sample. The HPLC chromatograms of freeze-dried samples of control solutions (water only, no fungus) containing disulfide **220** showed that it was not stable and spontaneously oxidized to sulfonic acid **222**. Nonetheless, the transformation of disulfide **220** in fungal cultures was slightly faster than in control medium solutions, indicating that this transformation might be partly mediated by an enzyme (**Figure 2.42**).

222
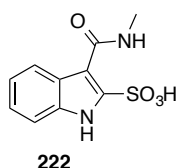
Figure 2.41 Structure of acid **222** from transformation of rutalexin (**5**) by *Alternaria brassicicola.*
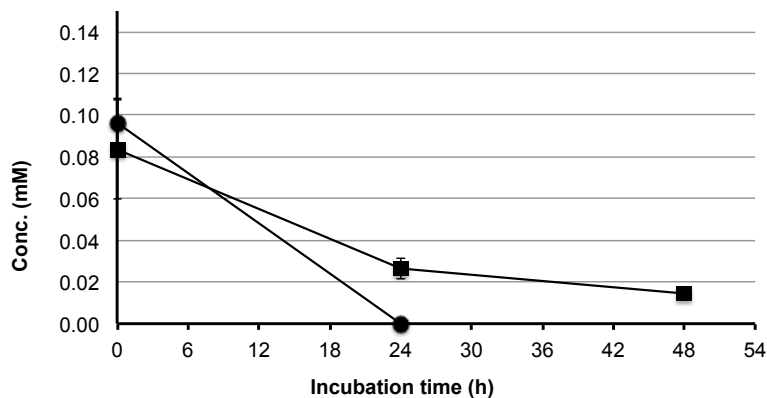
Figure 2.42 Progress curves of transformation of disulfide **220** (●) by *Alternaria brassicicola* and recovery of disulfide **220** (■) in control medium.

Comparison of the chromatograms of neutral extracts of cultures of *A. brassicicola* incubated with rutalexin (**5**) and control cultures (no rutalexin (**5**)) showed that phomapyrone G (**75**) was produced in higher concentration in cultures incubated with rutalexin (**5**) than in controls (**Figure 2.43**).
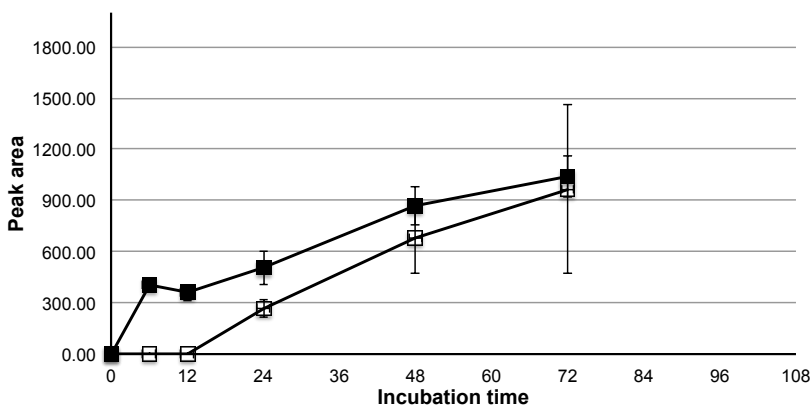
Figure 2.43 Progress curves of formation of phomapyrone G (**75**) in culture of *Alternaria brassicicola* incubated with rutalexin (**5**) (■) and in control culture (□).

2.1.3.3 Synthesis and antifungal activity of metabolites

Disulfide **220** and sulfonic acid **222** were obtained as summarized in **Scheme 2.21**. Disulfide **220** was synthesized by oxidative dimerization of sulfanylamide **18** followed by deprotection in 20% TFA in DCM as described in **Section 3.4.3.2**. Sulfonic acid **222** was synthesized from *m*-CPBA oxidation of disulfide **220** in MeOH in moderate yield (**Scheme 2.21**).
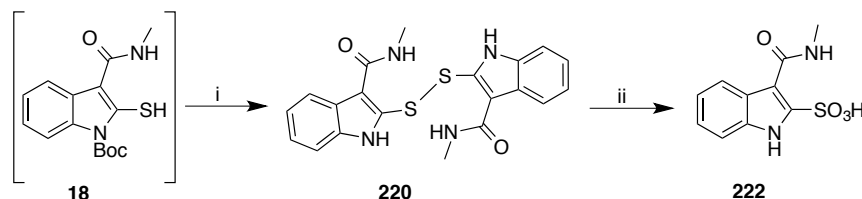
Scheme 2.21 Syntheses of **220** and **222**. Reagents and conditions: (i) TFA (20%) in DCM, r.t., 4 h, 80% (from **18**); (ii) *m*-CPBA, MeOH, r.t., 2.5 h, 59% (from **220**).

To establish if the biotransformation of rutalexin (**5**) by *A. brassicicola* was a detoxification reaction, mycelial radial growth assays were carried out. The antifungal activities against *A. brassicicola* of all metabolites resulting from transformation of rutalexin (**5**) were determined as described in **Section 3.2**; results of the assays are shown in **Table 2.10**.

Table 2.10 Antifungal activity of metabolites **220**, **222, 221** and synthetic compound **218** against *Alternaria brassicicola*.

Compound (#)	Inhibition ± SD (%)[a]		
	0.50 mM	**0.20 mM**	**0.10 mM**
2,2'-disulfanediylbis(N-methyl-1*H*-indole-3-carboxamide) **(220)**	n. d.	32 ± 0[b,c]	25 ± 3[c,d,e]
Adduct **221**	34 ± 3[b,c]	21 ± 3[d,e]	6 ± 3[f]
3-(methylcarbamoyl)-1*H*-indole-2-sulfonic acid **(222)**	5 ± 2[f]	n. i.	n. i.
N-methyl-2-(methylthio)-1*H*-indole-3-carboxamide **(218)**	29 ± 3[b,c,d]	20 ± 3[d,e]	15 ± 4[e]

[a]The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth on amended/growth in control) × 100]; values are averages of three independent experiments conducted in triplicate; n. i. = no inhibition, n. d. = not determined . For statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted α set at 0.05; n = 3; different letters in the same column ([b]–[f]) indicate significant differences (P < 0.05).

Comparison of the antifungal activities (**Table 2.8** and **Table 2.10**) suggested that growth inhibition gradually decreased from disulfide **220** to sulfonic acid **222**. However, disulfide **220** showed almost similar antifungal activity against *A. brassicicola* compared to rutalexin (**5**). At the highest concentration (0.20 mM), disulfide **220** showed 32% inhibitory activity that decreased to 25% at 0.10 mM. Disulfide **220** was not soluble in MM at concentrations higher than 0.20 mM. Sulfonic acid **222** did not show inhibitory activity against *A. brassicicola* at 0.20 and 0.10 mM. Results of antifungal activity of metabolites from biotransformation of rutalexin (**5**) indicated that the metabolism of rutalexin (**5**) by *A. brassicicola* is a detoxification.

2.1.3.4 Discussion and conclusion

Synthesis of rutalexin (**5**) was first reported by Pedras and co-workers in 2004 (Pedras et al., 2004b) and recently by Budovská and co-workers in 2015 (Budovská et al., 2015). The procedure for synthesis of rutalexin (**5**) was modified compared to the previously reported procedure by Pedras and co-workers (**Scheme 2.22**) (Pedras et al., 2004b) and the overall yield was improved compared to both reported procedures (Budovská et al., 2015; Pedras et al.,

2004b) (**Scheme 2.20**). In the previously reported procedure (Pedras et al., 2004b) the step containing reaction of sulfanylamide **18** with phosgene (step **iv**) afforded **19** in low yield (30% overall yield for two steps) (**Scheme 2.22**). In this work it was discovered that sulfanylamide **18** is not stable and spontaneously oxidized to disulfide **219** upon acidic extraction (Pedras et al., 2004b), for this reason methyl chloroformate was added directly to the reaction mixture (**Scheme 2.20**).
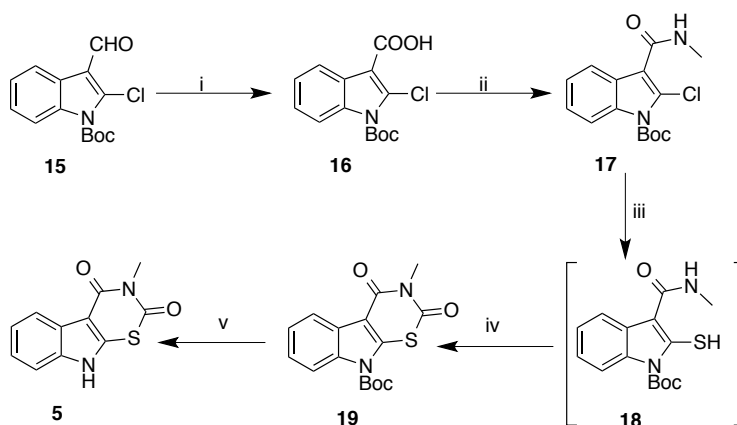
Scheme 2.22 Synthesis of rutalexin (**5**). Reagents and conditions: (i) NaClO$_2$, 2-methylbut-2-ene, *tert*- butyl alcohol/H$_2$O, r.t., 2 h, 96%; (ii) SOCl$_2$, THF, rt, 3 h, then CH$_3$NH$_2$, THF, 0 °C, 20 min, 85% (based on acid **16**); (iii) NaSH, DMF/H$_2$O, 0 °C, 2 h; (iv) NaH, THF, 0 °C, 10 min then COCl$_2$ (20% in toluene), -78 °C to rt, 4 h, 30% (based on **17**); (v) 165- 170 °C, 30 min, 100% (Pedras et al., 2004b).

After optimization of this reaction (**v**) compound **216** was obtained in 95% yield (**Scheme 2.20**).

Due to the poor solubility of rutalexin (**5**) in agar, its antifungal activity was not determined using radial mycelial growth assay. Previously, the antifungal activity of **5** was determined using a TLC bioassay using *C. cucumerinum* (Pedras et al., 2004b). In this work the inhibitory activity of rutalexin (**5**) against mycelial growth of *A. brassicicola* at 0.10 mM was determined using mycelial growth assay on MM, instead of potato dextrose agar (PDA). It was important to determine the antifungal activity of rutalexin (**5**) for concentrations higher than 0.10 mM. For this reason different experiments such as using different organic solvents or increasing the amounts of DMSO in the assay media were carried out, but the problem was not solved. That

is, concentrations higher than 0.10 mM could not be achieved due to precipitation. Bioassay of rutalexin (**5**) against *A. brassicicola* in MM showed that at 0.10 mM it is not a strong mycelial growth inhibitor (**Table 2.8**), but metabolites **221** and **222** resulting from transformation of rutalexin (**5**) showed lower inhibitory activity against *A. brassicicola* than rutalexin (**5**) (**Table 2.10**).

Rutalexin (**5**) was transformed to disulfide **220** and adduct **221** by *A. brassicicola*. Disulfide **220** was previously synthesized using a different procedure and was used to inhibit tyrosine kinase activity of EGFR (epidermal growth factor receptor) (Palmer et al., 1995). Disulfide **220** appeared to be oxidized further to sulfonic acid **222** by *A. brassicicola*, however additional work to prove this is an enzyme mediated transformation is required (Scheme **2.25**). From the structure of **220** and **221** it is likely that *A. brassicicola* transformed rutalexin (**5**) to amide **223** (**Scheme 2.23**), however **223** was not detected in the extracts of cultures incubated with rutalexin (**5**) either in water or MM. Compound **223** did not accumulate in cultures perhaps due to its reactivity, as it spontaneously oxidized to disulfide **220**. Its reaction with phomapyrone G (**75**) to yield a stable adduct **221** has no precedent. This is the first reported reaction between a phytoalexin metabolite and a secondary metabolite produced by a fungus. The pathway of rutalexin (**5**) transformation to sulfanylamide **18**, followed by oxidation to disulfide **220** or reaction with phomapyrone G (**75**) is proposed in **Scheme 2.23**.
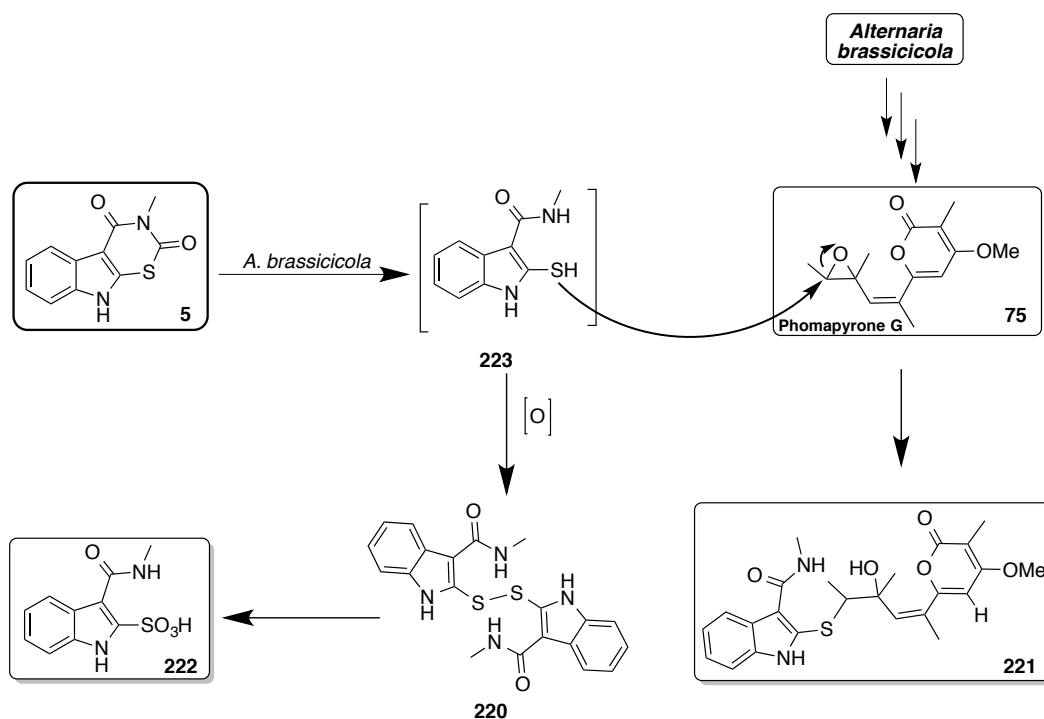
Scheme 2.23 Proposed transformation pathway of rutalexin (**5**) by *Alternaria brassicicola* and metabolic products.

In conclusion, rutalexin (**5**) was synthesized using an efficient procedure in reasonable overall yield and its biological activity against the cruciferous pathogen *A. brassicicola* was evaluated. This is the first report of transformation of rutalexin (**5**) by a plant pathogen. It was shown that the plant pathogen *A. brassicicola* was able to quickly detoxify rutalexin (**5**) to disulfide **220** and adduct **221**. Disulfide **220** was transformed further to sulfonic acid **222**, the least antifungal compound. The weak inhibitory activity of rutalexin (**5**) together with quick detoxification by *A. brassicicola* suggest that rutalexin (**5**) is not a phytoalexin of interest to improve the disease resistance of crucifers to *A. brassicicola*. Nonetheless, rutalexin (**5**) may have stranger roles in protecting crucifers against other fungal pathogens. Investigation of transformation of rutalexin (**5**) by other cruciferous pathogens is of interest to clarify the function of rutalexin (**5**) in protecting crucifers.

2.1.4 Rapalexin A, brassilexin and 1-methylbrassilexin

Brassilexin (**7**) is a phytoalexin produced mainly by *Brassica species* and biosynthesized from cyclobrassinin (**4**) (Pedras et al., 2011b). Brassilexin (**7**) was isolated and characterized in 1988 from the leaves of mustard (*Brassica juncea*) (Devys et al., 1988; Pedras et al., 2011b). Rapalexin A (**8**) is the first naturally occurring aromatic isothiocyanate that has been isolated from canola leaves (*Brassica rapa*), infected by *A. candida* (Pedras et al., 2011b; 2007b). 1-Methylbrassilexin (**165**) is not a natural product; it was synthesized by methylation of brassilexin (**7**).
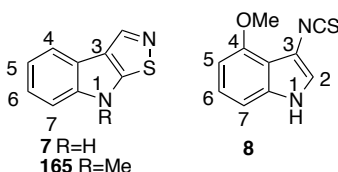
Figure 2.44   Structures of brassilexin (**7**), 1-methylbrassilexin (**165**) and rapalexin A (**8**).

2.1.4.1   Synthesis and antifungal activity

Brassilexin (**7**) (Pedras and Jha, 2005) and 1-methylbrassilexin (**165**) (Pedras and Jha, 2005) were synthesized as previously reported (Pedras et al., 2011b) and described in **Section 3.3.5**. The antifungal activities of rapalexin A (**8**), brassilexin (**7**) and 1-methylbrassilexin (**165**) were determined against *A. brassicicola* employing a mycelial radial growth assay (potato dextrose agar, PDA), as described in the experimental chapter **Section 4.4**. Different concentrations (0.50, 0.20, and 0.10 mM) of each compound in PDA were used for determination of the antifungal activity. Results of these assays are shown in **Table 2.11**.

Table 2.11 Antifungal activity of brassilexins **7**, **165** and rapalexin A (**8**) against *Alternaria brassicicola*.

Compound (#)	Inhibition ± SD (%)[a]		
	0.50 mM	0.20 mM	0.10 mM
Brassilexin (**7**)	100 ± 0^b	$79 \pm 4^{d,e}$	66 ± 4^f
1-Methylbrassilexin (**165**)	100 ± 0^b	$90 \pm 0^{b,c}$	$69 \pm 0^{e,f}$
Rapalexin A (**8**)	100 ± 0^b	$89 \pm 2^{c,d}$	66 ± 3^f

[a]The percentage of inhibition was calculated using the formula: % inhibition = 100 – [(growth on amended/growth in control) × 100]; values are averages of three independent experiments conducted in triplicate. For statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted α set at 0.05; n = 6; different letters in the same column ([b]–[f]) indicate significant differences (P < 0.05).

Results of the antifungal assays indicated that rapalexin A (**8**), brassilexin (**7**) and 1-methylbrassilexin (**165**) completely inhibited the mycelial growth of *A. brassicicola* at 0.50 mM (**Table 2.11**). Brassilexin (**7**) and rapalexin A (**8**) displayed similar activities causing ca. 70% inhibition at 0.10 mM, whereas 1-methylbrassilexin (**165**) was slightly more inhibitory than brassilexin (**7**) at 0.20 and 0.10 mM.

2.1.4.2   *Biotransformation of brassilexin, 1-methylbrassilexin and rapalexin A*

The transformation of brassilexin (**7**) and 1-methylbrassilexin (**165**) by *A. brassicicola* was investigated. Due to the inhibitory activity of brassilexin (**7**) and 1-methylbrassilexin (**165**) against *A. brassicicola* (**Table 2.11**), the metabolic studies were carried out using lowest tested concentration (0.10 mM) of brassilexins **7** and **165**. Cultures were incubated for different periods of time and samples were collected from each culture immediately after addition of brassilexin (**7**) up to 2 days. Samples of cultures were extracted and analyzed by HPLC-DAD-ESI-MS. Control solutions containing brassilexin (**7**) (no fungus) were analyzed similarly to determine its chemical stability during the incubation time.
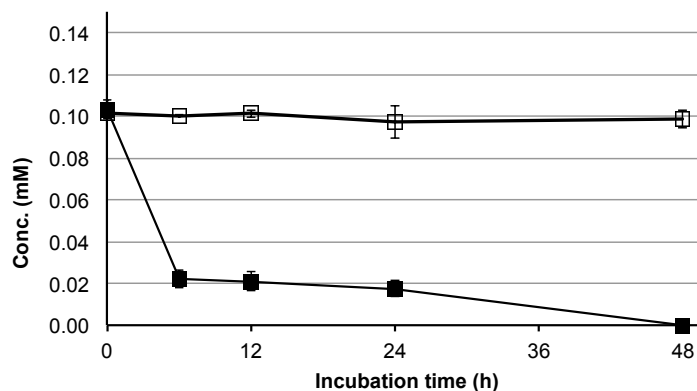
Figure 2.45 Progress curves of transformation of brassilexin (**7**, ■) by *Alternaria brassicicola* and recovery of brassilexin (**7**, □) in minimal medium.

The HPLC chromatograms of the neutral extracts of cultures of *A. brassicicola* incubated with brassilexin (**7**) showed that after 48 h (**Figure 2.45**) it was not detected. The chromatograms of the neutral extracts obtained after 12 h of incubation displayed the peak of brassilexin (**7**) and additional peaks at 6.2, 3.8 and 1.2 min. The peaks at 6.2, 3.8 and 1.2 min were not detected in either control cultures without brassilexin (**7**) or MM incubated with brassilexin (**7**). No additional peaks were detected either in acidic or basic extracts. The peaks at 6.2 and 3.8 min detected in the neutral extracts were due to 3-(aminomethylene)indoline-2-thione (**99**, t_R= 6.2 min), isatin (**224**, t_R = 3.8 min) (**Figure 2.46**), as determined by direct comparison with synthetic **99** and commercially available isatin (**224**). The metabolite responsible for the peak at 1.2 min was not available in our UV or MS data library. The HPLC-ESI-MS (negative mode) analysis of this peak indicated an ion at *m/z* 148 [M-H]⁻, which were 2 units higher than that of isatin (**224**). Based on this negative ion metabolite was proposed to contain two additional hydrogen atoms. This metabolite was proposed to be 3-hydroxyindoline-2-one (**226**) (**Figure 2.46**) and its structure was confirmed by direct comparison with an authentic synthetic sample.
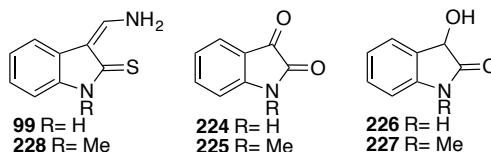
Figure 2.46 Structures of metabolites from transformations of brassilexin (**7**) and 1-methylbrassilexin (**165**) by *Alternaria brassicicola*.

Next, to establish the sequence of steps of brassilexin (**7**) transformation by *A. brassicicola*, each metabolite was administrated separately to cultures of *A. brassicicola*. The cultures were incubated, extracted and analyzed by HPLC-DAD-ESI-MS, as described above for brassilexin (**7**). The HPLC chromatograms of the neutral extracts of the cultures incubated with 3-(aminomethylene)indoline-2-thione (**99**) showed brassilexin (**7**) and 3-hydroxyindoline-2-one (**226**) (**Figure 2.47**). As previously reported, 3-(aminomethylene)-indoline-2-thione (**99**) was not stable in media and spontaneously oxidized to brassilexin (**7**) (Pedras and Suchy, 2005); however, its transformation in cultures was faster, indicating that an enzyme-mediated transformation occurred simultaneously (**Figure 2.47**). Because of the spontaneous oxidation of enamine **99** to brassilexin (**7**) determination of the amount of enamine (**99**) in each sample is not accurate. Due to presence of brassilexin (**7**) in the sample, making the calibration curve using the pure 3-(aminomethylene)-indoline-2-thione (**99**) was not achievable. Therefore, the amount of enamine in the controls and cultures was calculated as follows. Since the molecular masses of brassilexin (**7**) and enamine **99** are close (174 and 176 respectively), the concentration of enamine **99** was calculated in the control samples (no fungus) considering the amount of brassilexin (**7**) in that sample. The number of mmols for brassilexin (**7**) in each sample was calculated using calibration curve of brassilexin (**7**). Amount (mmols) of brassilexin (**7**) was subtracted from 0.10 mmol (the total number of mmols were added) to find the number of mmols for enamine **99** in each sample. The calibration curve for enamine **99** was prepared using these concentrations and related peak area in data points of control samples. Then the amount of enamine **99** in the cultures was obtained using this calibration curve (**Figure 2.47**).
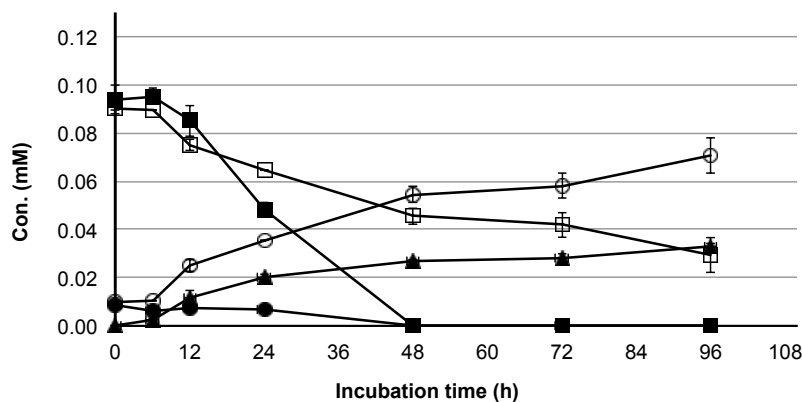
Figure 2.47   Progress curves of transformation of 3-(aminomethylene)-indoline-2-thione (**99**, ■) by *Alternaria brassicicola*, formation of brassilexin (**7**, ●), 3-hydroxyindoline-2-one (**226**, ▲), and recovery of 3-(aminomethylene)-indoline-2-thione (**99**, □) and brassilexin (**7**, ○) in minimal medium.

HPLC-DAD-ESI-MS analyses of the extracts of the cultures of *A. brassicicola* incubated with isatin (**224**) showed that it was transformed in ca. 3 h to 3-hydroxyindoline-2-one (**226**) by *A. brassicicola* (**Figure 2.48**). 3-Hydroxyindoline-2-one (**226**) was stable in cultures of *A. brassicicola* (**Scheme 2.25**) (**Figure 2.48**).
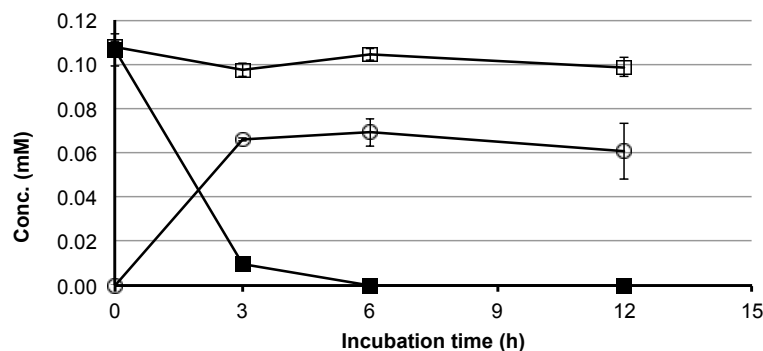
Figure 2.48   Progress curves of transformation of isatin (**224**, ■) by *Alternaria brassicicola*, formation of 3-hydroxyindoline-2-one (**226**, ○) and recovery of isatin (**224**, □) in minimal medium.

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Similar biotransformation experiments using 1-methylbrassilexin (**165**) showed that it was metabolized at similar rate *via* similar intermediates by *A. brassicicola*. 1-Methylbrassilexin (**165**) was metabolized to corresponding 1-methyl-3-hydroxyindoline-2-one (**227**) *via* enamine **228** and 1-methylisatin (**225**) (**Figure 2.49**, **Scheme 2.25**).
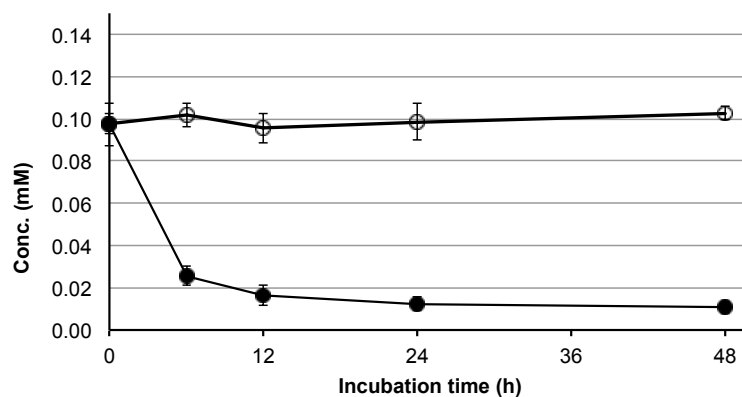
Figure 2.49   Progress curves of transformation of 1-methylbrassilexin (**165**, ●) by *Alternaria brassicicola* and recovery of methylbrassilexin (**165**, ○) in minimal medium.

Similar calculation, as described for enamine **99**, was employed to calculate the concentration of enamine **228** in the cultures of *A. brassicicola* incubated with 3-(aminomethylene)-1-methylindoline-2-thione (**228**) (**Figure 2.50**).
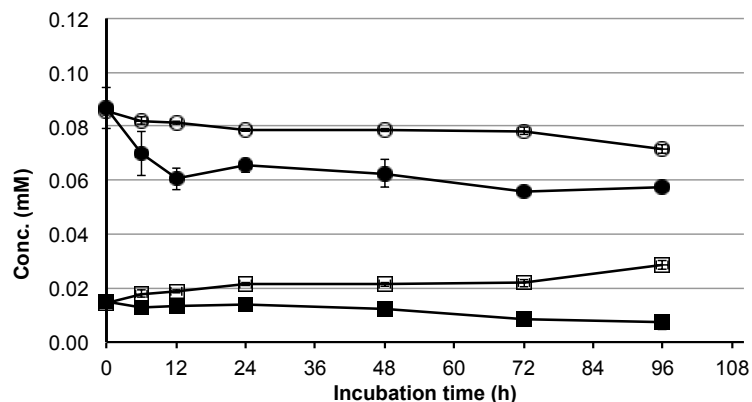
Figure 2.50 Progress curves of transformation of 3-(aminomethylene)-1-methylindoline-2-thione (**228**, ●) by *Alternaria brassicicola* and recovery of 1-methylbrassilexin (**165**, ■) from the cultures, 3-(aminomethylene)-1-methylindoline-2-thione (**228**, ○), and 1-methylbrassilexin (**165**, □) in minimal medium.

As it is shown in **Figure 2.47** at 48 h ca. 50% of enamine **99** was oxidized to brassilexin (**7**). In comparison, enamine **228** showed to be more stable in MM and less than 10% of **228** was oxidized to 1-methylbrassilexin (**165**) within 48 h (**Figure 2.50**). Also, enamine **228** was detected in the control extracts even after 48 h. The rate of transformation of brassilexin (**7**) and 1-methylbrassilexin (**165**) by *A. brassicicola* are comparable (ca. 48 h).
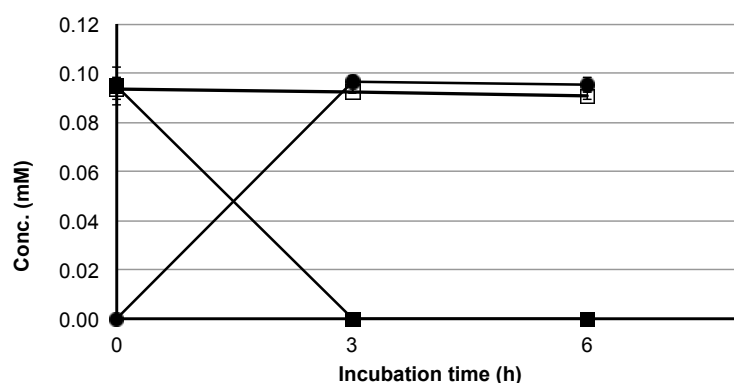
Figure 2.51 Progress curves of transformation of 1-methylisatin (**225**, ■) by *Alternaria brassicicola*, formation of 1-methyl-3-hydroxyindoline-2-one (**227**, ●), and recovery of 1-methylisatin (**225**, □) in minimal medium.

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The transformation of rapalexin A (**8**) by *A. brassicicola* was investigated similar to what was described for brassilexin (**7**). Due to the inhibitory activity of rapalexin A (**8**) against *A. brassicicola* (**Table 2.11**), the metabolic studies were carried out using 0.10 mM of rapalexin A (**8**) in MM. The HPLC-DAD-ESI-MS of neutral, basic and acidic extracts revealed that rapalexin A (**8**) remained in cultures for the duration of the incubation, that is, *A. brassicicola* did not transform rapalexin A (**8**) (**Figure 2.52**).
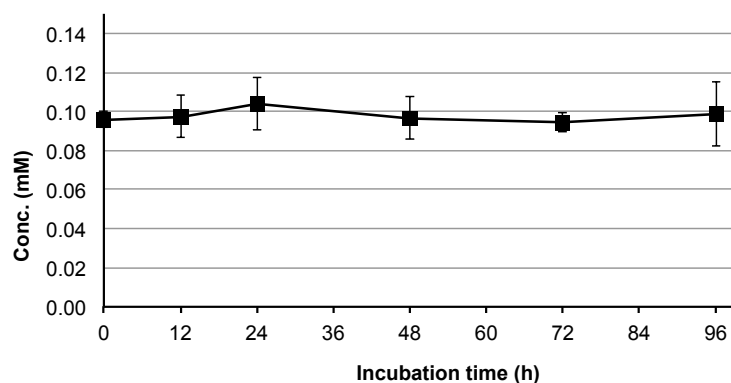
Figure 2.52   Progress curve of recovery of rapalexin A (**8**, ■) by *Alternaria brassicicola*.

2.1.4.3 *Synthesis and antifungal activity of metabolites*

Enamines **99** and **228** that resulted from transformation of brassilexin (**7**) and 1-methylbrassilexin (**165**) respectively, were synthesized following a published procedure (Pedras et al., 2011b), as described in **Section 3.4.4.2**. Metabolites **226** and **227** were synthesized in reasonable yield from reduction of isatin (**224**) and 1-methylisatin (**225**) using $NaBH_4$ in ethanol at room temperature (**Scheme 2.24**) (Bergonzini and Melchiorre, 2011).

224 R=H
225 R=Me

226 R=H
227 R=Me
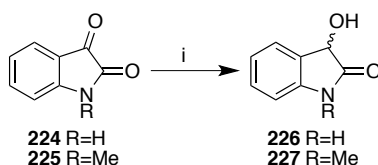
Scheme 2.24 Syntheses of 3-hydroxyindoline-2-one (**226**) and 1-methyl-3-hydroxyindoline-2-one (**227**). Reagents and conditions: (i) NaBH$_4$, 95% EtOH, r.t., 20 min, 68% for **226** and 92% for **227** (Bergonzini and Melchiorre, 2011).

To establish if the transformation of brassilexin (**7**) and 1-methylbrassilexin (**165**) by *A. brassicicola* were detoxification reactions, mycelial radial growth assays were carried out. The antifungal activities against *A. brassicicola* of all metabolites were determined as described in **Section 3.2**; results of these assays are shown in **Table 2.12**.

Table 2.12 Antifungal activity of compounds **224**-**227** against *Alternaria brassicicola*.

Compound (#)	Inhibition ± SD (%)[a]		
	0.50 mM	**0.20 mM**	**0.10 mM**
Isatin (**224**)	63 ± 0[b]	27 ± 3[d,e]	15 ± 4[g,h]
Methylisatin (**225**)	32 ± 0[d]	18 ± 4[f,g]	10 ± 4[h,i]
3-hydroxyindoline-2-one (**226**)	46 ± 0[c]	24 ± 4[e,f]	n. i.
3-hydroxy-1-methylindoline-2-one (**227**)	14 ± 3[g,h]	6 ± 3[i,j]	n. i.

[a] The percentage of inhibition was calculated using the formula: % inhibition = 100 − [(growth on amended/growth in control) × 100]; values are averages of three independent experiments conducted in triplicate; n. i. = no inhibition. For statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted α set at 0.05; n = 6; different letters in the same column ([b–j]) indicate significant differences (P < 0.05).

Comparison of the antifungal activities (**Table 2.12**) suggested that growth inhibition gradually decreased from isatins **224** and **225** to 3-hydroxyindoline-2-ones **226** and **227** and that inhibition caused by compounds **226** and **227** was weaker than that caused by brassilexins **7** and **165**. At highest concentration (0.50 mM) of isatin (**224**) the mycelial growth of *A. brassicicola*

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was inhibited 63%, while 3-hydroxyindoline-2-one (**226**) caused 46% inhibition at identical concentration (0.50 mM). Compounds **226** and **227** showed no inhibitory activity against mycelial growth of *A. brassicicola* at the lowest tested concentrations (0.10 mM) (**Table 2.12**). The antifungal activity of enamines **99** and **228** were not determined because of the low stability of samples in media (spontaneous oxidation to corresponding brassilexins).

2.1.4.4   Discussion and conclusion

Brassilexin (**7**), 1-methylbrassilexin (**165**) and rapalexin A (**8**) displayed strong antifungal activity against mycelial growth of *A. brassicicola*. However, they were not as strong as camalexin (**1**) with 100% growth inhibition for both 0.50 and 0.20 mM concentrations. At the highest concentration (0.50 mM) all tested compounds could completely inhibit the mycelial growth of *A. brassicicola*. Compared to 1-methylbrassilexins (**165**), brassilexin (**7**) and rapalexin A (**8**) were slightly weaker against mycelial growth of *A. brassicicola* especially at the lowest tested concentration (0.10 mM) (**Table 2.11**). Comparison of the antifungal activities of metabolites with parent compounds (**Table 2.11** and **Table 2.12**) suggested that the growth inhibition gradually decreased from isatin (**224**) and 1-methylisatin (**225**) to 3-hydroxyindoline-2-one (**226**) and 3-hydroxy-1-methylindoline-2-one (**227**) that were weaker than their parent compounds. Results of antifungal activities indicated that the transformations of brassilexin (**7**) and 1-methylbrassilexin (**165**) by *A. brassicicola* are detoxifications.

Brassilexin (**7**) and 1-methylbrassilexin (**165**) were metabolized by *A. brassicicola* to enamines **99** and **228** at a similar rate (ca. 96 h) likely using enzymatic reduction of N-S bonds of their isothiazole rings. Enamines **99** and **228** were transformed further by *A. brassicicola* to isatin (**224**) and 1-methylisatin (**225**) respectively. Isatins **224** and **225** were reduced to 3-hydroxyindoline-2-one (**226**) and 1-methyl-3-hydroxyindoline-2-one (**227**). The pathway of transformation of brassilexins by *A. brassicicola* is proposed in **Scheme 2.25**.
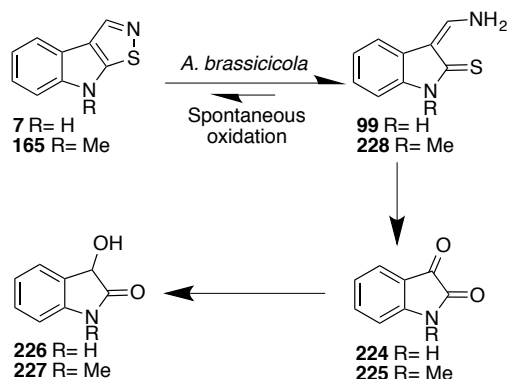
Scheme 2.25 Biotransformations of brassilexin (**7**) and 1-methylbrassilexin (**165**) by *Alternaria brassicicola* and metabolic products.

Previous work showed that brassilexin (**7**) was transformed to enamine **99** by *L. maculans* (Pedras and Suchy, 2005). However, the rate and complete pathway of transformations were different.

Bioassay with rapalexin A (**8**) against *A. brassicicola* showed that, mycelial growth was completely inhibited at highest concentration (0.50 mM). Furthermore, it was shown that rapalexin A (**8**) is resistant to transformation by *A. brassicicola*. Previously it was investigated that rapalexin A (**8**) was also resistant to transformation by *L. maculans* (Pedras and Sarma-Mamillapalle, 2012) with strong antifungal activity against *L. maculans* (Pedras and Sarma-Mamillapalle, 2012).

In conclusion, it was shown for the first time that the plant pathogen *A. brassicicola* was able to detoxify brassilexin (**7**) and 1-methylbrassilexin (**165**) quickly (ca. 96 h, $t_{1/2}$<6 h) using similar transformation pathways, involving enzymatic reduction of N-S bond of their isothiazole rings of brassilexins. Enamines were metabolized further to corresponding alcohols **226** and **227**, the least inhibitory among the metabolic products, *via* isatin (**224**) and 1-methylisatin (**225**) respectively. Previously it has been shown that brassilexin (**7**) and 1-methylbrassilexin (**165**) could inhibit (93% and 73% respectively at 0.30 mM) the fungal enzyme cyclobrassinin hydrolase (CHAb) from *A. brassicicola* (Pedras and Minic, 2014), which catalyzes detoxification of cyclobrassinin (**4**) by *A. brassicicola*. These results indicated that brassilexin (**7**) is not of

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interest as a lead structure to design paldoxins against *A. brassicicola* because it is metabolized quickly by this plant pathogen.

Rapalexin A (**8**) showed strong antifungal activity against *A. brassicicola* and was also resistant to transformation by this fungus. Since rapalexin A (**8**) was not metabolized by *A. brassicicola* and *L. maculans* (Pedras and Sarma-Mamillapalle, 2012) and showed strong antifungal activity against both pathogens, accumulation of rapalexin A (**8**) into cultivated *Brassica* species could be great in protection of them against both black spot and blackleg diseases.

2.2 Inhibitory activity of camalexin analogues against brassinin oxidase of *Leptosphaeria maculans*

Previous work (Pedras, 2014; Pedras et al., 2011b; 2008a) suggested that *L. maculans* produces inducible brassinin oxidase (BO) that can detoxify brassinin (**9**), one of the most important cruciferous phytoalexins. Due to the importance of brassinin (**9**) production in crucifers (Pedras, 2014), inhibition of its detoxification could be used to control *L. maculans*. As depicted in **Scheme 2.26** the detoxification of brassinin (**9**) by *L. maculans*, involves the oxidative transformation of a dithiocarbamate to an aldehyde catalyzed by BO (**Scheme 2.26**) (Pedras et al., 2008a).
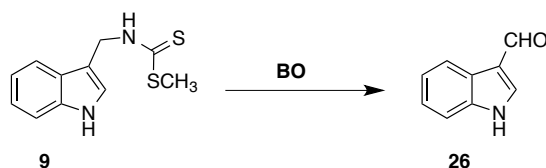
Scheme 2.26 Detoxification of the phytoalexin brassinin (**9**) catalyzed by brassinin oxidase (BO).

In previous work amongst several synthetic compounds and a few phytoalexins, camalexin (**1**) was shown to be a strong inhibitor of brassinin oxidase (30% at 0.10 mM and 53%

at 0.30 mM) (Pedras et al., 2008a). Camalexin (**1**) is not biosynthesized in cultivated crucifers and is not metabolized by *L. maculans*. For this reason, camalexin (**1**) was considered as a lead structure for development of phytoalexin detoxification inhibitors against *L. maculans*. A group of compounds were designed and synthesized involving replacement of the thiazolyl moiety, modification of the indolyl moiety, replacement of indolyl with naphthyl and phenyl substituents and replacement of both indole and thiazolyl moieties (Pedras et al., 2009b). Among all tested compounds, camalexins with substitution at the C-5 and C-6 showed the strongest inhibitory activity against BO (Pedras et al., 2009b). As was described in **Section 2.1.1** compounds **167-170**, **173**, **174**, **184** and **185** were synthesized to better understand the mechanism of oxidative degradation of thiazole ring of camalexin (**1**) in camalexins transformation by *A. brassicicola*. However, the effect of substitution(s) on thiazole ring of camalexin (**1**) on the activity against BO was not determined. Having these camalexin related structures in hand and considering the previous work (Pedras et al., 2009b), it was of interest to evaluate their activities against BO (**Figure 2.53**).
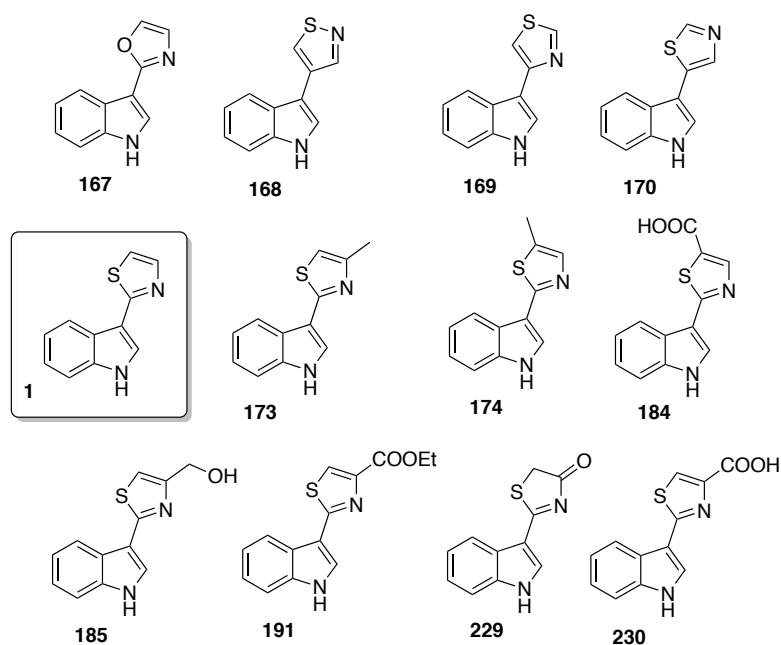
Figure 2.53   Potential brassinin oxidase inhibitors with structures based on camalexin (**1**).

2.2.1 Synthesis of potential inhibitors

Camalexin (**1**) and related structures **167-170**, **173**, **174**, **184**, **185**, **229** and **230** were synthesized and their inhibitory activity against BO was investigated using cell-free extracts (**Table 2.14**). Synthesis of all analogues except **229** and **230** is described in **Section 2.1.1**(Pedras and Abdoli, 2013).
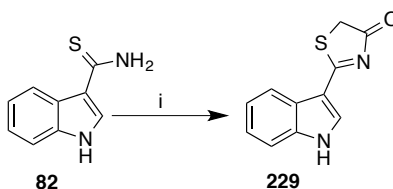
Scheme 2.27 Synthesis of **229**. Reagents and condition: (i) Ethyl 2-chloro-3-oxopropanoate, 95% EtOH, H$_2$SO$_4$, 90 °C, 2 h, 58%.

2-(1*H*-indol-3-yl)oxazol-4(5H)-one (**229**) was prepared from indole-3-thiocarboxamide (**82**) as shown in **Scheme 2.27**. Condensation of thiocarboxamide **82** with ethyl 2-chloro-3-oxopropanoate in acidic conditions gave compound **229** in 58% yield**.**
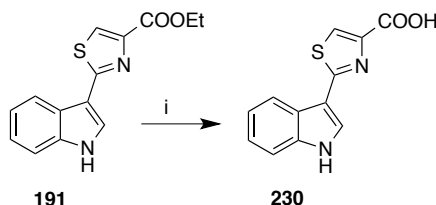
Scheme 2.28 Synthesis of acid **230**. Reagent and condition: (i) NaOH, THF, 14 h, r.t., 89% (Moody et al., 1997).

Acid **230** was obtained from basic hydrolysis of ester **191** using sodium hydroxide as shown in **Scheme 2.28**. The reaction was carried out at room temperature for 14 h (Moody et al., 1997). The preparation of ethyl ester **191** is described in **Section 2.1.1.4**.

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2.2.2 Determination of the inhibitory activity

The effect of the phytoalexin camalexin (**1**) and related structures **167-170**, **173**, **174**, **184**, **185**, **229** and **230** on BO activity were evaluated using two different concentrations (0.10 mM and 0.30 mM). In this work cell-free extracts of *L. maculans* containing BO were used for inhibitory activity determination of potential inhibitors. It was established that production of BO in fungal cultures required induction with specific compounds (Pedras et al., 2008a; 2005). Cell-free extracts of control cultures of *L. maculans* showed residual activity, while the BO activity was ca. 2.31 mU/mg (U = μmol.min^{-1}) for the cultures incubated with 3-phenylindole (**234**) (Pedras et al., 2008a). Furthermore, it was shown that camalexin (**1**) induced detoxification of brassinin (**9**) in cultures of *L. maculans* (Pedras et al., 2005). That is, BO activity was detected in crude cell-free extracts only when *L. maculans* was grown in presence of inducers such as 3-phenylindole (**234**) or camalexin (**1**) (Pedras et al., 2008a). Cell-free extracts were prepared using a previously published procedure (Pedras et al., 2009b) described in detail in **Section 3.5.2**. In brief, cultures of *L. maculans* were grown in MM and after 48 h of incubation 3-phenylindole (**234**) (final concentration, 0.10 mM) in CH_3CN was added to induce production of BO. After incubation for an additional 24 h, the mycelia were collected by filtration and stored at -20 °C. Frozen mycelial cells were homogenized in ice-cold extraction buffer at 4 °C using a mortar and pestle. The protein homogenate was obtained by centrifugation of the mixture. The cell-free extracts obtained from the previous step were dialyzed at 4 °C (described in **Section 3.5.2**). The dialyzed cell-free extracts were used to determine BO activity. The Bradford protein assay was used to quantify the amount of protein in cell-free extracts (using bovine serum albumin) as described in **Section 3.5.3**. The specific activity of cell-free extracts was defined as the amount (nmol) of indole-3-carboxaldehyde (**26**) formed per min per mg of protein. The amount of product was determined after extraction of the reaction mixture and HPLC analysis of the extracts. It was established that the presence of an electron acceptor is essential for activity of BO (Pedras et al., 2008a). BO could accept a wide range of cofactors, including phenazine methosulfate (PMS) (Pedras et al., 2008a). The reaction mixture contained assay buffer (diethanolamine (DEA), dithiothreitol (DTT), Triton X-100 and deionized water), brassinin (**9**), PMS, deionized water and cell-free extracts in a total volume of 1000 μl. After incubation of the reaction mixture at 24 °C and solvent extraction, HPLC was used for detection and quantification

of indole-3-carboxaldehyde (**26**). The concentration of indole-3-carboxaldehyde (**26**) was determined using a calibration curve prepared from pure indole-3-carboxaldehyde (**26**). To establish if storage of cell-free extracts at -20 °C affected the specific activity of BO, enzyme assays of both dialyzed and non-dialyzed cell-free extracts were tested, as summarized in **Table 2.13**. The amount of protein in dialyzed cell-free extracts was lower than in non-dialyzed cell-free extracts (**Table 2.13**).

Table 2.13   Specific activity of brassinin oxidase (BO) in cell-free extracts of mycelia of *Leptosphaeria maculans*.

Time (Days)[c]	Amount of product 26 (nmol)[a]		Amount of protein (µg in 50 µl of cell-free extracts)		Specific activity of BO (nmol/mg/min)[b] ± SD	
	Dialyzed	Non-dialyzed	Dialyzed	Non-dialyzed	Dialyzed	Non-dialyzed
0	n. d.	n. d.	70.2 ± 1.2	148.8 ± 4.3	n. d.	n. d.
5	43.0 ± 2.0	n. d.	70.6 ± 0.90	148.8 ± 3.8	22.8 ± 1.1	n. d.
9	52.0 ± 1.2	70 ± 20	94.5 ± 0.10	175.9 ± 5.8	27.6 ± 0.7	19.6 ± 0.60
13	48.6 ± 0.80	72 ± 40	100.2 ± 0.90	142.9 ± 0.40	24.6 ± 0.4	25.2 ± 1.4
15	54.3 ± 1.4	89 ± 30	98.0 ± 0.50	144.3 ± 1.5	27.7 ± 0.7	30.7 ± 1.0
32	50.6 ± 0.40	80 ± 60	100.2 ± 0.70	150.4 ± 2.5	25.3 ± 0.2	26.6 ± 2.1

[a] nmols of indole-3-carboxaldehyde (**26**) were determined using a calibration curve prepared from pure indole-3-carboxaldehyde (**26**).
[b] Results are expressed as means and standard deviation of triplicate samples of cell-free extracts of one set of cultures; brassinin at 0.10 mM, n. d. = not determined.
[c] Days stored at -20 °C

Camalexin (**1**) and related structure **167-170**, **173**, **174**, **184**, **185**, **229** and **230** were tested for potential inhibition of BO as follows. First, the stability of each compound was tested under identical reaction conditions. All compounds were found to be stable within the incubation period (20 min) except compound **229** that partially decomposed to more than one compound. The assays were done in triplicate using cell-free extracts from one set of cultures. Each potential inhibitor (final concentration 0.10 and 0.30 mM) dissolved in DMSO was added to a vial

containing PMS (0.10 mM) and brassinin (**9**, 0.10 mM) followed by addition of assay buffer (DEA (20 mM), DTT (0.10 mM), 0.1% triton X-100, deionized water), deionized water and finally 50 μl of cell-free extract. The reaction mixture was incubated at room temperature (24 °C) for 20 min. Then samples were immediately extracted separately with EtOAc and the extracts were analyzed by HPLC for the detection and quantification of the reaction product, indole-3-carboxaldehyde (**26**). Control experiments containing only brassinin (**9**, 0.10 mM) were carried out similarly. Results of the enzymatic assays are summarized in **Table 2.14**.

Table 2.14   Effect of compounds on brassinin oxidase (BO) in dialyzed cell-free extracts of mycelia of *Leptosphaeria maculans*.

Substrate (#)[a] +Inhibitor (#)	Specific activity[b,d] (nmol/mg/min) ± SD		Inhibition (%)[c,d]	
	0.10 mM	0.30 mM	0.10 mM	0.30 mM
Brassinin (**9**) + Camalexin (**1**)	14.7 ± 0.9	9.3 ±0.5	42 ± 4	64 ± 2
Brassinin (**9**) + 5′-methylcamalexin (**174**)	18.3 ± 0.5	15.1 ± 0.9	29 ± 2	41 ± 3
Brassinin (**9**) + 4-(1*H*-indol-3-yl)thiazole (**169**)	22.7 ± 0.3	19.0 ± 0.7	11 ± 1	26 ± 3
Brassinin (**9**) + 2-(1*H*-indol-3-yl)oxazole (**167**)	20.7 ± 0.2	16.6 ± 1.3	19 ± 1	35 ± 5
Brassinin (**9**) + (2-(1*H*-indol-3-yl)thiazol-4-yl)methanol (**185**)	21.6 ± 0.7	16.9 ± 0.8	16 ± 3	34 ± 3
Brassinin (**9**) + 4-(1*H*-indol-3-yl)isothiazole (**168**)	22.2 ± 1.0	14.1 ± 0.7	13 ± 4	45 ± 2
Brassinin (**9**) + 5-(1*H*-indol-3-yl)thiazole (**170**)	18.0 ± 0.7	15.5 ± 0.6	30 ± 3	39 ± 2
Brassinin (**9**) + 4′-methylcamalexin (**173**)	20.4 ± 1.5	20.1 ± 0.9	20 ± 6	21 ± 3
Brassinin (**9**) + 2-(1*H*-indol-3-yl)oxazol-4(5*H*)-one (**229**)	22.4 ± 0.6	18.1 ± 1.3	13 ± 2	29 ± 5
Brassinin (**9**) + 2-(1*H*-indol-3-yl)thiazole-5-carboxylic acid (**184**)	27.3 ± 1.0	26.3 ± 2.2	n. i.	n. i.
Brassinin (**9**) + 2-(1*H*-indol-3-yl)thiazole-4-carboxylic acid (**230**)	25.5 ± 0.4	24.1 ± 0.7	n. i.	6 ± 2
Brassinin (**9**) + Ethyl 2-(1*H*-indol-3-yl)thiazole-4-carboxylate (**191**)	23.5 ± 0.7	15.3 ± 1.2	8 ± 3	40 ± 4

[a]Substrate (brassinin (**9**)) was used at 0.10 mM in all experiments.

[b]BO activity was measured under standard conditions (described in **section 4.6.3**) in the presence of potential inhibitors (0.10 and 0.30 mM) and brassinin (**9**, 0.10 mM). Specific activity of control assays (26 ± 2 nmol/mg/min).

[c]Inhibition is expressed as a percentage of activity of control (100%), n. i. = no inhibition.

% inhibition = 100 – [(amount of aldehyde in assay/amount of aldehyde in control) × 100];

Among all tested 5′- methylcamalexin (**174**, ca. 29% at 0.10 mM and 41% at 0.30 mM) was the most potent inhibitors of BO activity, followed by 4-(1*H*-indol-3-yl)isothiazole (**168**, ca. 13% at 0.10 mM and 45% at 0.30 mM) and ethyl 2-(1*H*-indol-3-yl)thiazole-4-carboxylate (**191**, 8% at 0.10 mM and 40% at 0.30 mM). 2-(1*H*-indol-3-yl)thiazole-5-carboxylic acid (**184**) and 2-(1*H*-indol-3-yl)thiazole-4-carboxylic acid (**230**) (6% at 0.30 mM) did not show any activity against BO. 4-(1*H*-indol-3-yl)isothiazole (**168**) showed strong inhibitory activity (45% at 0.30 mM) against BO, while at 0.10 mM the activity was very low. In addition, 2-(1*H*-indol-3-yl)oxazole (**167**), (2-(1*H*-indol-3-yl)thiazol-4-yl)methanol (**185**) and 2-(1*H*-indol-3-yl)oxazol-4(5*H*)-one (**229**) showed very similar inhibitory activity against BO (**Table 2.14**).

2.2.3 Discussion and conclusion

It was reported that camalexin (**1**) and some related structures with substitution at C-5 and C-6 of indole could inhibit the activity of BO and slow down the transformation of brassinin (**9**) to indole-3-carboxaldehyde (**26**) (Pedras et al., 2009b). In continuation of that work, a new group of camalexin related structures that were synthesized by modification of the thiazole ring were tested as potential inhibitors of BO (**Figure 2.53**). The specific activity of BO and the amount of protein of dialyzed and non-dialyzed cell-free extracts were determined after storage at -20 °C for different times (**Table 2.13**). The amount of protein present in dialyzed cell-free extracts was lower than that for non-dialyzed sample, suggesting protein losses during dialysis. In addition, the results showed that dialyzed cell-free extracts had similar specific activity compared to non-dialyzed cell-free extracts (**Table 2.13**).

None of the tested camalexin related compounds showed activity stronger than camalexin (**1**) against BO. Based on the percentage of inhibition caused by **174**, **184** and **230**, increasing the size of substituents at C-4′ and C-5′ decreases the inhibitory activity of the potential inhibitors, however additional structures need to be tested to confirm this hypothesis. Interestingly, almost all 4′-substituted compounds were active against BOLm, however it was very weak for 2-(1*H*-

indol-3-yl)thiazole-4-carboxylic acid (**230**). These results together with previous results (Pedras et al., 2009b) indicated that the camalexin scaffold is a reasonable model to design potential inhibitors of BO. Furthermore, camalexin (**1**) is resistant to metabolism by *L. maculans* (Pedras et al., 1998), however, the transformation of these inhibitors by *L. maculans* needs to be investigated. Nevertheless, camalexin showed potential to be a good lead structure for synthesis of inhibitors to inhibit the activity of BO from *L. maculans*. These compounds cannot be considered PALDOXINS, due to their strong antifungal activity against most cruciferous pathogens (Pedras et al., 2011b; Pedras and Abdoli, 2013).

In conclusion, however almost all related structures of camalexin (**1**), compounds **173, 13-15**, **18**, **31**, **32**, **56**, **57** and **214**, showed inhibitory activity against BO, but none of them was stronger than camalexin (**1**).

2.3 General conclusion and future direction

The antifungal activity of camalexin (**1**), 1-methylcamalexin (**2**), 6-methoxycamalexin (**3**) and related structures **167-174** towards the *A. brassicicola* was determined (**Table 2.1**). They were found to be strongly active against mycelial growth of *A. brassicicola* (Pedras and Abdoli, 2013). While, brassilexin (**7**), 1-methylbrassilexin (**165**) and rapalexin A (**8**) showed strong antifungal activity against *A. brassicicola* but they were not as strong as camalexin (**1**) (**Table 2.11**). Cyclobrassinin (**4**), 1-methylcyclobrassinin (**193**) and rutalexin (**5**) showed moderate antifungal activity against *A. brassicicola* (**Table 2.8**, **Table 2.3**).

It was established that rapalexin A (**8**) could resist transformation by *A. brassicicola*. While, camalexin (**1**), 1-methylcamalexin (**2**) and 6-methoxycamalexin (**3**) were slowly (ca. 10 days) metabolized by *A. brassicicola* (ca. 10 days) (Pedras and Abdoli, 2013). This slow detoxification of camalexin (**1**) indicates its importance in the resistance of *A. thaliana* to *A. brassicicola* (Thomma et al., 1999). Modification of the thiazolyl moiety of camalexin (**1**) prevents degradation of the thiazole ring in related structures **173**, **174** and **168-170**. The transformation of analogues **173** and **174** revealed that substitution at C-4′ or C-5′ of thiazolyl rings of compounds **173** and **174** blocked the degradation of the thiazole ring by *A. brassicicola*, however, this did not prevent oxidation of the methyl group on compounds **173** and **174**. The resistance of compounds **168**, **169** and **170** to transformation by *A. brassicicola* in comparison to the transformation that occurs in oxazole **167**, diazoles **171**, **172** and camalexins **1**, **2** and **3** indicates that oxidation of thiazolyl ring by *A. brassicicola* required attachment to the indole ring by C-2′ (Pedras and Abdoli, 2013). Cyclobrassinin (**4**) and 1-methylcyclobrassinin (**193**) were detoxified very quickly compared to camalexin (**1**) (8 h *vs* 10 days) by *A. brassicicola*. Cyclobrassinin (**4**) has been discovered to inhibit BHAb (Pedras et al., 2012) however, the quick transformation of cyclobrassinin (**4**) by *A. brassicicola* revealed that it is not a good lead structure to use in synthesizing inhibitors of BHAb. Brassilexin (**7**) and 1-methylbrassilexin (**165**) were metabolized by *A. brassicicola* faster than camalexin (**1**) (24 h *vs* 10 days). Brassilexin (**7**) has been discovered to inhibit CHAb (Pedras and Minic, 2014), however, its metabolism by *A. brassicicola* shows that structure of brassilexin (**7**) cannot be used as a model in the development of CHAb inhibitors. Metabolism of rutalexin (**5**) by plant pathogens is reported here for the first time. Rutalexin (**5**) is proposed to be metabolized by *A. brassicicola* to

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an unstable metabolite, sulfanylamide **223**, which stabilizes with oxidation to disulfide **75** or reacts with phomapyrone G (**75**), a secondary metabolites from *A. brassicicola*, to form stable adduct **75**.

All metabolites resulting from metabolism of phytoalexins and their *N*-methylated derivatives by *A. brassicicola* showed lower inhibitory activity against *A. brassicicola* compare to their parent compounds which means that complete fungal transformation of all tested phytoalexins by *A. brassicicola* are detoxifications.

Potential inhibitors of brassinin oxidase, **167-170**, **173**, **174**, **184**, **185**, **229** and **230**, were synthesized based on the phytoalexin camalexin (**1**) scaffold with modification of its thiazole ring. Compounds **167-170**, **173**, **174**, **184**, **185**, **229** and **230** were used for the inhibition of BO using the mycelial cell-free extracts of *L. maculans* containing BO. Most of the selected compounds were able to inhibit the activity of BO. However, none of them were as active as camalexin (**1**) in inhibiting of BO. Overall, compounds **168**, **174**, **170**, **191** and **167** showed substantial effects in the inhibition of BO, and compound **168** was found to be the strongest amongst all tested compounds. Further studies are required to understand the antifungal activity of all tested compounds against *L. maculans* as well as their metabolic stability in *L. maculans*.

Future work

o Design, synthesize and evaluate the effect of compounds derived from other potential heterocyclic skeletons (not indolyl) in the activity of BOLm.

o Investigation of transformation of rutalexin (**5**) by other cruciferous pathogens.

o Determine the antifungal activity of compounds **1**, **167-170**, **173**, **174**, **184**, **185**, **191**, **229** and **230** against *L. maculans*.

o Determine the metabolic stability of compounds **1**, **167-170**, **173**, **174**, **184**, **185**, **191**, **229** and **230** in *L. maculans*.

3   EXPERIMENTAL

3.1   General

All reagents and chemicals were purchased either from Sigma-Aldrich or Alfa Aesar. All solvents were HPLC grade, except those used in synthetic procedure. If necessary solvents were dried prior to use according to established procedures (pyridine and DMF with 3 Å molecular sieves, THF and Et$_2$O over sodium and benzophenone, DCM, CH$_3$CN, and benzene over CaH$_2$). Organic extracts were dried over Na$_2$SO$_4$ and solvents were removed under reduced pressure in a rotary evaporator.

Reaction process was monitored using thin layer chromatography (TLC). TLC was carried out on alumina sheets pre-coated with silica gel, Merck, 60 F$_{254}$ (20 × 20 cm × 0.25 mm). Compounds developed on the TLC plates were visualized under UV light (254/366 nm) and/or by dipping in a solution of 5% (w/v) aqueous phosphomolybdic acid containing 1% (w/v) ceric sulphate and 4% (w/v) H$_2$SO$_4$, followed by charring on hot plate at 200 °C.

Flash column chromatography (FCC) was carried out using silica gel grade 60, mesh size 230-400 Å.

NMR spectra were obtained on Bruker Avance 500 or 600 MHz spectrometers. For ^{1}H NMR (500 or 600 MHz) and ^{13}C NMR (125.8 MHz), the chemical shift values (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS). For ^{1}H NMR (500 or 600 MHz) the δ values were referenced to CDCl$_3$ (CHCl$_3$ at 7.27 ppm), CD$_3$CN (CHD$_2$CN at 1.94 ppm), CD$_3$OD (CHD$_2$OD at 3.31) or (CD$_3$)$_2$SO (DMSO-d_5 at 2.50). Multiplicities are indicated by the following notations: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and br = broad. Spin coupling (*J* values) are reported to the nearest 0.5 Hz. For ^{13}C NMR (125.8 MHz) the δ values are referenced to CDCl$_3$ (77.23 ppm), CD$_3$CN (118.69 ppm), CD$_3$OD (49.15 ppm), (CD$_3$)$_2$SO (39.50 ppm).

Fourier transform infrared (FTIR) data were acquired on Bio-Rad FTS-40 spectrometers. Spectra were measured by the diffuse reflectance method on samples dispersed in KBr.

HPLC analysis was carried out with Agilent high performance liquid chromatography instruments equipped with quaternary pump, automatic injector, diode array detector (DAD,

wavelength range 190–600 nm), degasser, and a column having an in-line filter. Several elution methods were used:

Method A: column Zorbax Eclipse XDB-C18 (5 μm particle size silica, 4.6 × 150 mm), mobile phase H_2O–MeOH (1:1, v/v) to MeOH, for 25.0 min, linear gradient, and at a flow rate 0.75 ml/min;

Method B: column Zorbax SB-C18 (3.5 μm particle size silica, 3 × 100 mm), mobile phase H_2O–MeOH (1:1, v/v) to MeOH, for 35.0 min, linear gradient, and at a flow rate 0.40 ml/min;

Method C: column Zorbax SB-C18 (3.5 μm particle size silica, 3.0 × 100 mm), mobile phase H_2O–MeOH (7:3, v/v) to MeOH, for 35.0 min, linear gradient, at a flow rate of 0.40 ml/min;

Method D: column Zorbax SB-C18 (3.5 μm particle size silica, 3.0 × 100 mm), mobile phase H_2O–MeOH (9.50:0.50, v/v) to MeOH, for 35.0 min, linear gradient, at a flow rate of 0.40 ml/min;

Method E: column Zorbax SB-C18 (3.5 μm particle size silica, 3.0 × 100 mm), mobile phase H_2O–CH_3CN (1:0, v/v) to (1:9, v/v), for 28.0 min, linear gradient, at a flow rate of 0.40 ml/min.

HPLC-ESI-MS analysis was carried out with an Agilent 1100 series HPLC system equipped with an auto sampler, binary pump, degasser, and a diode array detector connected directly to a mass detector (Agilent G2446A MSD-Trap-XCT ion trap mass spectrometer) with an electrospray ionization (ESI) source. Chromatographic separations were carried out at room temperature using Eclipse XDB-C 18 column (5 mm particle size silica, 150 × 4.6 mm I.D.). The mobile phase consisted of a linear gradient of:

Method F: H_2O (with 0.20% HCO_2H)-CH_3CN (with 0.20% HCO_2H) from 75:25 to 25:75 in 35 min, to 0:100 in 5 min and a flow rate of 1.0 ml/min.

Method G: H_2O (with 0.20% HCO_2H)-CH_3CN (with 0.20% HCO_2H) from 90:10 to 0:100 in 35 min and a flow rate of 1.0 ml/min.

Data acquisition was carried out in positive and negative polarity modes in a single LC run, and data processing carried out with Agilent Chemstation Software. Samples were dissolved in CH_3CN, MeOH, or mixture of H_2O-MeOH (50:50).

MS [high resolution (HR), electron ionization (EI)] were obtained on a VG 70 SE and Jeol AccuToF 4G GCv mass spectrometers.

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Minimal media (MM) is a mixture of glucose (15 g/l, 83.3 mM) and 4 different solutions as mentioned below. Glucose, solution 1 and solution 3 were mixed in distilled water and autoclaved. Sterilized solution 2 and 4 were added to the above mixture at room temperature.

Solution 1: KNO_3 (3.1 g/l, 31 mM), K_2HPO_4 (0.75 g/l, 4.3 mM), KH_2PO_4 (0.75 g/l, 5.5 mM), NaCl (0.10 g/l, 1.7 mM), asparagine (0.28 g/l, 2.1 mM);

Solution 2: $CaCl_2 \cdot 2H_2O$ (0.10 g/l, 0.68 mM), $MgSO_4 \cdot 7H_2O$ (0.50 g/l, 2.0 mM);

Solution 3: $ZnSO_4.7H_2O$ (0.40 mg/l, 1.4 µg), $CuSO_4.5H_2O$ (0.079 mg/l, 0.32 µM), $MnSO_4.4H_2O$ (0.041 mg/l, 0.18 µM), MoO_3 (85%, 0.050 mg/l, 0.12 µM), ferric citrate (0.038 mg/l, 0.10 µM), $Na_2B_4O_7.10H_2O$ (0.04 mg/l, 0.10 µM);

Solution 4: Thiamine (0.10 mg/l, 0.38 µM);

3.2   Fungal isolates and antifungal activity

Alternaria brassicicola (UAMH 7474) and *L. maculans* (UAMH 9410) were obtained from the University of Alberta Microfungus Collection and Herbarium. *A. brassicicola* was grown on potato dextrose agar (PDA) plates at room temperature (23 ± 1 °C), under continuous light for 15 days. Spores suspensions of fungus was prepared overlaying the PDA plates with 10 ml of sterile distilled water, and the plate surfaces were rubbed with a flamed glass rod. The suspension was filtered and transferred to falcon tubes and the spores were separated by centrifugation at 3000g for 30 min. After one washing with sterile distilled water and separation by centrifugation, the spores were counted under a microscope using haemocytometer and stored at -20 °C. Similarly, spores of *L. maculans* were collected from the cultures of the fungus on V8 agar [20% (v/v) V8 juice, 0.75 g/l $CaCO_3$, 100 mg/l streptomycin sulfate, 40 mg/l Rose Bengal, 15 g/l agar] plates (Pedras and Khan, 1996).

Antifungal activity

The antifungal activity of compounds against *A. brassicicola* was determined using a mycelial radial growth bioassay. *A. brassicicola* was grown on PDA plates for 7 days at 23 ºC under constant light. Sterile culture plates (12-well, 20 mm diameter) were used in all bioassays.

A solution of compounds to be tested (dissolved in DMSO) was added to PDA or MM (up to 1% DMSO in final volume) to prepare the different concentrations (0.50, 0.20 and 0.10 mM). Control plates were prepared to contain same percentage of DMSO as it is in the fungus with compound in PDA or fungus with compound in MM. Plates containing compound and control (1.5 ml PDA or 1 ml MM per well) were inoculated with mycelium plugs [2 mm diameter, cut from the edge of 7-day old solid culture of *A. brassicicola*] placed upside down on the center of each well and incubated under constant light. Mycelial growth in each well was measured and % inhibition values were calculated as previously reported (% inhibition=100–[(growth on amended/growth in control) × 100]) (Pedras et al., 2009a). All bioassay experiments were carried out in triplicate, at least two times.

3.3 Synthesis of phytoalexins and related structures

3.3.1 Camalexins

3.3.1.1 *Camalexin (1) and 6-methoxycamalexin (3)*
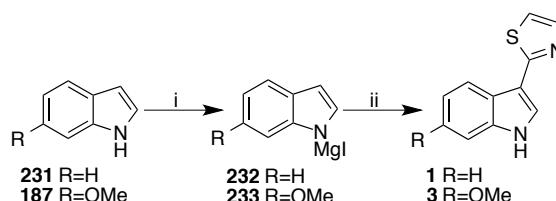
Scheme 3.1 Syntheses of camalexin (**1**) and 6-methoxycmalexin (**3**). Reagents and conditions: (i) MeI, Mg, Et$_2$O, r.t.; (ii) 2-Bromothiazole, Benzene, 24 h, 90 °C, 80% (**1**) and 56% (**3**) (Ayer et al., 1992)(Pedras et al., 2011b).

Methyl iodide (0.25 ml, 4.0 mmol) was added to a mixture of Mg (98 mg, 4.0 mmol) in dry diethyl ether (2 ml) under argon at room temperature. After all magnesium was dissolved, diethyl ether was distilled off and dry benzene (2 ml) was added to the reaction mixture. A solution of indole (**231**, 445 mg, 3.80 mmol) in dry benzene (2 ml) was added dropwise to the

reaction mixture. The reaction mixture was stirred for additional 10 minutes, followed by addition of 2-bromothiazole (0.080 ml, 0.90 mmol) and was refluxed for 24 h. The reaction mixture was quenched with cold water and extracted with EtOAc. The combined extracts were washed with brine, dried and concentrated to dryness. The residue was subjected to FCC (silica gel, EtOAc-hexane, 3:7) to afford camalexin (**1**) in 80% yield as yellow solid (Ayer et al., 1992). Similarly, 6-methoxycamalexin (**3**) was synthesized in 56% yield as yellow solid using 6-methoxyindole (**187**, 150mg, 1.02mmol), Mg (48 mg), methyl iodide (0.19 ml, 3.0 mmol) and 2-bromothiazole (0.19 ml, 2.1 mmol).

Camalexin (1)

Melting point: 145–146 °C

HPLC t_R = 12.3 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max}(nm): 215, 275, 315.

^{1}H NMR (500 MHz, CDCl$_3$): δ 9 (b, 1H), 8.3 (d, J = 7 Hz, 1H), 7.9 (s, 1H), 7.8 (s, 1H), 7.4 (d, J = 7 Hz, 1H), 7.3 (m, 3H).

6-Methoxycamalexin (3)

Melting point: 157–146 °C

HPLC t_R = 12.5 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max}(nm): 220, 290, 320.

^{1}H NMR (500 MHz, CDCl$_3$): δ 8.7 (br, 1H), 8.1 (d, J = 8.5 Hz, 1H), 7.8 (d, J = 3.5 Hz, 1H), 7.7 (d, J = 2.5 Hz, 1H), 7.2 (d, J = 3.5 Hz, 1H), 6.9 (dd, J = 2.5, 9 Hz, 1H), 6.8 (d, J = 2 Hz, 1H), 3.8 (s, 3H).

3.3.1.2   1-Methylcamalexin (2)
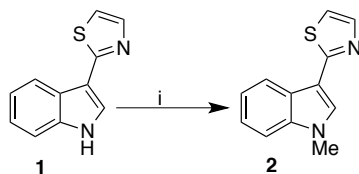
Scheme 3.2   Synthesis of 1-methylcamalexin (**2**). Reagents and conditions: (i) MeI, NaH, THF, 2 h, r.t., 100% (Pedras and Liu, 2004).

Sodium hydride 60% suspension in mineral oil (19 mg, 0.48 mmol, washed with hexane) was added to a solution of camalexin (**1**, 46 mg, 0.23 mmol) in dry THF (4 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 10 min, followed by dropwise addition of methyl iodide (0.020 ml, 0.32 mmol). The ice bath was removed and the reaction mixture was stirred at room temperature for additional two hours followed by dilution with water and extraction with EtOAc. The combined extracts were dried and concentrated to dryness. The residue was subjected to FCC (silica gel, EtOAc-hexane, 1:1) to afford 1-methylcamalexin (**2**) in quantitative yield as light yellow solid (Moody et al., 1997; Pedras and Liu, 2004).

Melting point: 69–70 °C

HPLC t_R = 15.0 min (method A).

UV (HPLC, CH_3OH-H_2O) λ_{max}(nm): 220, 270, 330.

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.3 (m, 1H), 7.8 (d, J = 3.5 Hz, 1H), 7.7 (d, J = 1.5 Hz, 1H), 7.3 (m, 3H), 7.2 (d, J = 3.5 Hz, 1H), 3.7 (s, 3H).

3.3.1.3 4′-Methylcamalexin (173)
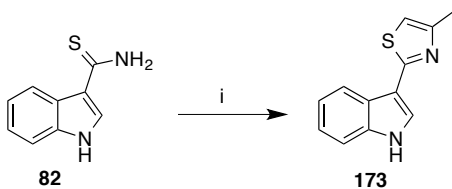
Scheme 3.3 Synthesis of 4′-methylcamalexin (**173**). Reagent and condition: (i) CH$_3$COCH$_2$Cl, 95% EtOH, 1.5 h, 80 °C, quantitative.

2-Chloropropanone (0.010 ml, 0.12 mmol) was added dropwise to a solution of indole-3-thiocarboxamide (**82**, 10 mg, 0.060 mmol) in EtOH (95%, 2 ml) and the reaction mixture was refluxed at 80 °C for 1.5 h. The reaction mixture was concentrated and the residue was diluted with H$_2$O. Aqueous phase was neutralized with NaHCO$_3$ (satd. sol.) and then was extracted with EtOAc. The combined extracts were dried and concentrated to dryness to afford 4′-methylcamalexin (**173**, 14 mg, quantitative) as white powder.

Melting point: 168–169 °C.

HPLC t_R = 15.8 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 220, 280, 320.

FTIR (KBr) ν_{max} cm^{-1}: 3119, 2222, 1534, 1445, 1242, 1116, 733.

^{1}H NMR (500 MHz, CDCl$_3$): δ 9.42 (br, 1H), 8.19 (d, J = 7.5 Hz, 1H), 7.88 (d, J = 2.5 Hz, 1H), 7.33 (d, J = 7.5 Hz, 1H), 7.25 (m, 2H), 6.79 (s, 1H), 2.50 (s, 3H).

^{13}C NMR (125.8 MHz, CDCl$_3$): δ 163.0, 152.2, 136.6, 124.9, 124.8, 123.2, 121.5, 120.0, 112.1, 112.0, 110.8, 17.2.

HRMS-EI m/z: measured 214.0561 ([M]$^+$, calcd. 214.0565 for C$_{12}$H$_{10}$N$_2$S) (100%).

3.3.1.4   5′-Methylcamalexin (174)
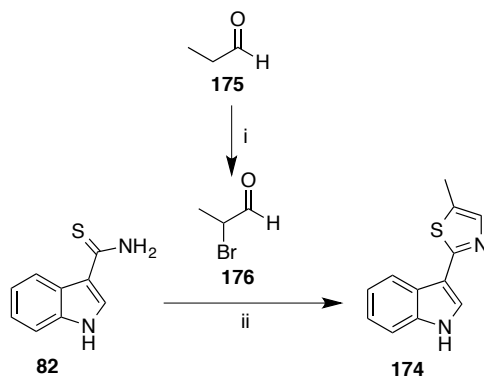
Scheme 3.4   Synthesis of 5′-methylcamalexin (**174**). Reagents and conditions: (i) Br$_2$, 1,4-dioxane, 1 h, 0 °C; (ii) CH$_3$CHBrCHO (**176**), 95% EtOH, 2 h, 80 °C, 55%.

Bromine (2.7 ml, 0.060 mmol) was added dropwise to a solution of propionaldehyde (**175**, 4.0 ml, 0.060 mmol) in 1,4-dioxane (0.50 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h followed by concentration to dryness. The residue was subjected to FCC (silica gel, EtOAc–hexane, 30:70) to afford 2-bromopropanal (**176**) (Gangjee et al., 2005).

2-Bromopropanal (**176**, 100 μl, 0.340 mmol) was added to a solution of indole-3-thiocarboxamide (**82**, 30 mg, 0.17 mmol) in EtOH (95%, 1.5 ml) and was refluxed at 80 °C for 2 h. The reaction mixture was diluted with water, neutralized with NaHCO$_3$ (satd. sol.), and extracted with CH$_2$Cl$_2$. The combined extracts were dried and concentrated to dryness. The residue was subjected to FCC (silica gel, EtOAc–hexane, 1:1) to yield 5′-methylcamalexin (**174**, 20 mg, 55%) as white powder.

Melting point: 170–172 °C.

HPLC t_R = 16.3 min (method A).

UV (HPLC, CH$_3$OH–H$_2$O) λ_{max} (nm): 220, 278, 320.

FTIR (KBr) ν_{max} cm^{-1}: 2833, 1540, 1451, 1242, 1119, 740.

^{1}H NMR (500 MHz, CDCl$_3$): δ 8.60 (br, 1H), 8.22 (m, 1H), 7.83 (d, J = 2.5 Hz 1H), 7.48 (s, 1H), 7.44 (m, 1H), 7.29 (m, 2H), 2.53 (s, 3H).

^{13}C NMR (500 MHz, CDCl$_3$): δ 162.1, 139.7, 136.5, 130.9, 124.6, 124.3, 123.0, 121.3, 120.4, 112.3, 111.7, 12.0.

HRMS-EI *m/z*: measured 214.0563 ([M]$^+$, calcd. 214.0565 for C$_{12}$H$_{10}$N$_2$S) (100%).

3.3.1.5   1H-3-(4′-Thiazolyl)indole (169)
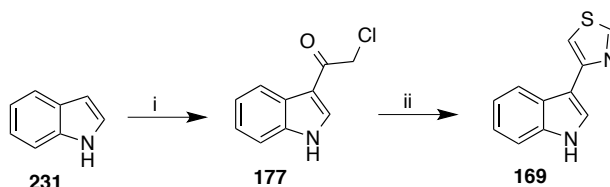
Scheme 3.5 Synthesis of 1H-3-(4′-thiazolyl)indole (**169**). Reagents and conditions: (i) Chloroacetylchloride, toluene, pyridine, 1 h, 60 °C and 1 h, r.t.; (ii) Thioformamide, 95% EtOH, 3 h, 80 °C, 38%.

Chloroacetylchloride (48 mg, 0.42 mmol) was added dropwise to a solution of indole (500 mg, 4.2 mmol) and pyridine (500 μl) in toluene (10 ml) at 60 °C. After complete addition of chloroacetylchloride, the reaction mixture was kept at 60 °C for additional 1 h. The reaction mixture was cooled to room temperature; H$_2$O (13 ml) and MeOH (2 ml) were added to the reaction mixture. The reaction mixture was stirred at room temperature for an additional 1 h and was concentrated using rotary evaporator. The resulting precipitate was filtered and was crystalized with EtOH. The crystals were washed with diethyl ether after filtration to afford 2-chloro-1-(1*H*-indol-3-yl)ethanone (**177**). Thioformamide (29 mg, 0.47 mmol) was added to the solution of 2-chloro-1-(1*H*-indol-3-yl)ethanone (**177**, 50 mg, 0.26 mmol) in EtOH (95%, 2 ml). The reaction mixture was kept at 90 °C for 3 h, then was diluted with water and extracted with EtOAc. The combined extracts were dried and concentrated to dryness. The residue was subjected to FCC (silica gel, EtOAc-hexane, 1:1) to afford 2-(1*H*-indol-3-yl)-5-methylthiazole (**169**) as light brown powder in 38% yield (20 mg, 0.10 mmol) (Moody et al., 1997).

Melting point: 140–141 °C.

HPLC t_R = 11.28 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 220, 278, 320.

FTIR (KBr) ν_{max}cm^{-1}: 3438, 3070, 1566, 1416, 1253, 1102, 990, 819.

^{1}H NMR (500 MHz, CD$_3$CN): δ 8.71 (s, 1H), 8.20 (br, 1H), 7.85 (d, 1H), 7.63 (s, 1H), 7.44 (m, 1H), 7.28 (s, 1H), 7.25 (d, 1H), 7.07 (m, 2H).

^{13}C NMR (500 MHz, CD$_3$CN): δ 154.1, 153.2, 138.2, 126.3, 125.9, 123.5, 121.5, 121.3, 113.1, 112.9, 110.8.

HRMS-EI *m/z:* measured 200.0402 ([M]$^+$, calcd. 200.0408 for C$_{11}$H$_8$N$_2$S) (100%).

3.3.1.6   1H-3-(5′-Thiazolyl)indole (170)
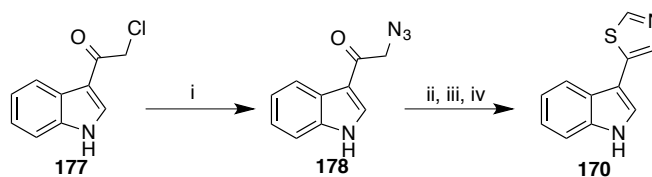
Scheme 3.6   Synthesis of 1H-3-(5′-thiazolyl)indole (**170**). Reagents and conditions: (i) NaN$_3$, H$_2$O, acetone, 20 h, 50 °C; (ii) Pd/C, H$_2$, HCl, MeOH, 3 h, r.t., 85%; (iii) CH$_3$CH$_2$OCHO, Et$_3$N, THF, 8 h, 50 °C, 86%; (iv) Lawesson's reagent, 1,4-dioxane, 30 min, 120 °C, 68%.

A mixture of 2-chloro-1-(1H-indol-3-yl)ethanone (**177**, 300 mg, 1.50 mmol) and sodium azide (200 mg, 3.00 mmol) in acetone (20 ml) and H$_2$O (10 ml) was heated at 50 °C for 20 h. The reaction mixture was cooled to room temperature, was diluted with H$_2$O (20 ml) and extracted with CH$_2$Cl$_2$. The combined extracts were dried and concentrated to dryness. The crude product was washed with CH$_2$Cl$_2$ and used for the next step (250 mg, 81%). Pd/C (50 mg) was added to a solution of 2-azido-1-indolyl-3-ethanone (**178**, 50 mg, 0.23 mmol) in MeOH (3 ml) followed by HCl (20 μl). The reaction flask was connected to an H$_2$-filled balloon and the reaction mixture was stirred at room temperature under H$_2$ atmosphere for 3 h. The reaction mixture was filtered and the filtrate was concentrated. The crude product was subjected to FCC (silica gel, MeOH-CH$_2$Cl$_2$, 2:8) to afford 2-amino-1-indolyl-3-ethanone (40 mg, 85%). Ethyl formate (1.5 ml) was added to a solution of 2-amino-1-indolyl-3-ethanone (30 mg, 0.16 mmol) in dry THF (2 ml) followed by Et$_3$N (50 μl). The reaction mixture was heated at 50 °C for 8 h, concentrated and diluted with H$_2$O. The mixture was neutralized with aqueous HCl (1 M) and

extracted with EtOAc. The combined extracts were dried, concentrated to dryness and the residue was subjected to FCC (silica gel, MeOH-CH$_2$Cl$_2$, 1:9) to afford N-(2-(1H-indol- 3-yl)-2-oxoethyl)formamide (30 mg, 86%). Lawesson's reagent (50 mg) was added to a solution of N-(2-(1H-indol-3-yl)-2-oxoeth- yl)formamide (30 mg, 0.15 mmol) in 1,4-dioxane (1.5 ml) while the solution was heating at 120 °C under argon. After 30 min, 1,4-dioxane was removed with a rotary evaporator and the residue was diluted with H$_2$O. The aqueous solution was neutralized using NaHCO$_3$ (satd. sol.) and extracted with EtOAc. The combined extracts were dried, concentrated to dryness and the residue was subjected to FCC (silica gel, EtOAc-hexane, 1:1) to afford 1H-3-(5′-thiazol- yl)indole (**170**, 20 mg, 68%) as white powder.

Melting point: 140–142 °C

HPLC t_R = 8.2 min (method A).

UV (HPLC, CH$_3$OH–H$_2$O) λ_{max} (nm): 210, 242, 262, 300.

FTIR (KBr) ν_{max} cm^{-1}: 3204, 1566, 1416, 1253, 823.

^{1}H NMR (500 MHz, CD$_3$CN): δ 9.64 (br, 1H), 8.76 (s, 1H), 8.10 (s, 1H), 7.87 (d, J = 8 Hz, 1H), 7.60 (d, J= 2.5 Hz 1H), 7.50 (d, J = 8 Hz 1H), 7.25 (dd, J= 8, 7 Hz, 1H), 7.20 (dd, J = 8, 7 Hz, 1H).

^{13}C NMR (500MHz, CD$_3$CN): δ 151.4, 139.1, 138.0, 134.1, 126.4, 125.4, 123.9, 121.8, 120.4, 113.3, 108.0.

HRMS-EI m/z: measured 200.0413 ([M]$^+$, calcd. 200.0408 for C$_{11}$H$_8$N$_2$S) (100%).

3.3.1.7 1H-3-(4′-Isothiazolyl)indole (168)
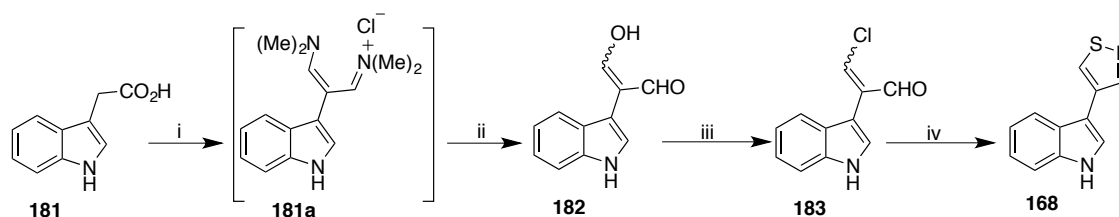
Scheme 3.7 Synthesis of 1H-3-(4′-isothiazolyl)indole (**168**). Reagents and conditions: (i) POCl$_3$, DMF; (ii) NaOH, 1,4-dioxane; (iii) SOCl$_2$, THF, 10 min, -20 °C; (iv) NH$_4$SCN, DMF, 12 h, 70 °C, 56%.

Indolyl-3-acetic acid (**181**, 220 mg, 1.25 mmol) in DMF (0.25 ml) was added to a mixture of DMF (0.50 ml) in $POCl_3$ (0.35 ml, 3.2 mmol) at 0 °C. The reaction mixture warmed up to room temperature and was refluxed at 90 °C for 3 h. The reaction mixture was cooled to room temperature, crushed ice was added to adjust the volume to ca. 30 ml and was extracted with CH_2Cl_2. The combined extracts were dried, and concentrated to dryness to afford crude diamine **181a** (315 mg) as a yellowish powder in 91% yield. Diamine **181a** (100 mg, 0.400 mmol) was dissolved in 1,4-dioxane (2 ml) followed by addition of NaOH (25% aq, 2 ml), and the reaction mixture was refluxed for 8 h. 1,4-Dioxane was removed with a rotary evaporator, the residue was diluted to ca. 30 ml by addition of crushed ice and acidified to pH ≤ 3 using aq HCl (1:1). The resulting mixture was extracted with diethyl ether, the combined extracts were dried, and the solvent was evaporated to yield crude enolaldehyde **182** (69 mg, 90%). $SOCl_2$ (0.35 ml, 4.8 mmol) was added to crude enolaldehyde **182** (50 mg, 0.26 mmol) in THF (1.5 ml) and the reaction mixture was cooled to -20 °C and stirred for 10 min. The solvent was evaporated and the residue was dissolved in EtOAc and subjected to FCC (silica gel, EtOAc-Hexane, 7:3) to yield a fraction containing chloroacrolein **183** that was immediately used in the next step. NH_4SCN (30 mg, 0.40 mmol) was added to the solution of chloroacrolein **183** (20 mg, 0.10 mmol) in DMF (1.5 ml), and the reaction mixture was heated at 70 °C for 12 h with stirring (caution: in hood, NaOH trap for HCN). The reaction mixture was diluted with brine (20 ml) and extracted with EtOAc. The combined extracts were dried, the solvent was evaporated, and the residue was subjected to FCC (silica gel, EtOAc–hexane, 3:7) to afford 1H-3-(4′ -isothiazolyl)indole (**168**, 16 mg, 56% yield) as light yellowish powder.

Melting point: 124–125 °C

HPLC t_R = 12.2 min (method A).

UV (HPLC, CH_3OH-H_2O) λ_{max} (nm): 225, 290.

FTIR (KBr) ν_{max} cm^{-1}: 3406, 1582, 1457, 1243, 890.

^{1}H NMR (500 MHz, CDCl$_3$): δ 8.83 (s, 1H), 8.73 (s, 1H), 8.36 (br, 1H), 7.86 (d, J = 7.5 Hz, 1H), 7.48 (s, 2H), 7.25 (m, 3H).

^{13}C NMR (125.8MHz, CDCl$_3$): δ 156.6, 140.6, 136.4, 133.3, 125.7, 122.9, 122.2, 120.8, 119.3, 111.6, 109.8.

HRMS-EI m/z: measured 200.0407 ([M]$^+$, calcd. 200.0408 for $C_{11}H_8N_2S$) (100%).

3.3.1.8 1H-3-(2′-Oxazolyl)indole (167)
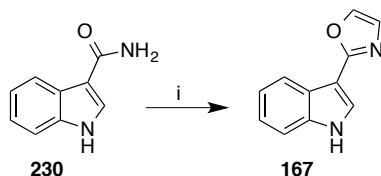
Scheme 3.8 Synthesis of 1H-3-(2′-oxazolyl)indole (**167**). Reagents and conditions: (i) Chloroacetaldehyde, 95% EtOH, 2 h, reflux, 73% (Ayer et al., 1992).

A solution of indole-3-carboxamide (**230**, 30 mg, 0.20 mmol) and chloroacetaldehyde (0.04 ml) in EtOH (95%, 0.7 ml) was refluxed for 2 h. EtOH was removed under vacuum using a rotary evaporator and then the reaction mixture was diluted with water and extracted with EtOAc. The combined extracts were dried and concentrated to dryness to afford 1H-3-(2′-oxazolyl)indole (**167**, 25 mg, 0.10 mmol, 73%) as light yellowish powder (Ayer et al., 1992).

Melting point: 130–132 °C

HPLC t_R = 10.1 min (method A).

UV (HPLC, CH_3OH-H_2O) λ_{max} (nm): 220, 260, 297.

FTIR (KBr) ν_{max} cm^{-1}: 1672, 1526, 1456, 1215, 1132, 742, 505.

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.65 (br, 1H), 8.3 (dd, J = 2, 3 Hz, 1H), 7.9 (d, J = 3 Hz, 1H), 7.7 (s, 1H), 7.4 (dd, J = 3, 2 Hz, 1H), 7.3 (m, 2H), 7.2 (s, 1H).

^{13}C NMR (125.8MHz, $CDCl_3$): δ 160.3, 136.9, 136.3, 127.6, 125.7, 124.8, 123.4, 121.7, 121.3, 111.7, 106.1.

HRMS-EI m/z: measured 184.0637 ([M]$^+$, calcd. 184.0637 for $C_{11}H_8N_2O$) (100%).

3.3.1.9 2-(1H-indol-3-yl)oxazol-4(5H)-one (229)
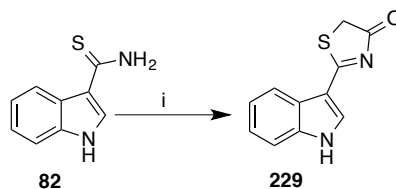
Scheme 3.9   Synthesis of 2-(1H-indol-3-yl)oxazol-4(5H)-one (**229**). Reagents and condition: (i) Ethyl 2-chloro-3-oxopropanoate, EtOH, H$_2$SO$_4$, 6 h, 90 °C, 58%.

Ethyl 2-chloro-3-oxopropanoate (245 mg, 1.30 mmol) was added to a solution of indole-3-thocarboxamide (**82**, 70 mg, 0.40 mmol) in EtOH (5 ml) followed by addition of H$_2$SO$_4$ (1 drop). The reaction mixture in sealed reaction vial was heated at 90 °C for 6 h. The reaction mixture was cooled to room temperature. EtOH was removed using a rotary evaporator; the reaction mixture was diluted with water and extracted with EtOAc. The combined extracts were dried and concentrated to dryness. The residue was subjected to FCC (silica gel, EtOAc-hexane, 1:1) to give pure **229** (50 mg, 0.20 mmol, 58%) as brown powder.

Melting point: 150–151 °C

HPLC t_R = 11.4 min (method C).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 210, 255, 270, 350.

FTIR (KBr) ν_{max} cm^{-1}: 3117, 1672, 1526, 1468, 1360, 1207, 1132, 738.

^{1}H NMR (500 MHz, DMSO-d_6): δ 12.6 (br, 1H), 8.6 (d, J = 3.5 Hz, 1H), 8.2 (dd, J = 2, 3.5 Hz, 1H), 7.6 (dd, J = 3.5, 2 Hz, 1H), 7.3 (m, 2H), 4.1 (s, 2H).

^{13}C NMR (125.8MHz, DMSO-d_6): δ 191.4, 188.1, 137.2, 134.9, 124.7, 123.6, 122.7, 121.2, 112.9, 111.0, 37.1.

HRMS-FD m/z: measured 216.03595 ([M]$^+$, calcd 216.03573 for C$_{11}$H$_8$N$_2$OS) (100%).

3.3.1.10 2-(1H-indol-3-yl)thiazole-4-carboxylic acid (230)
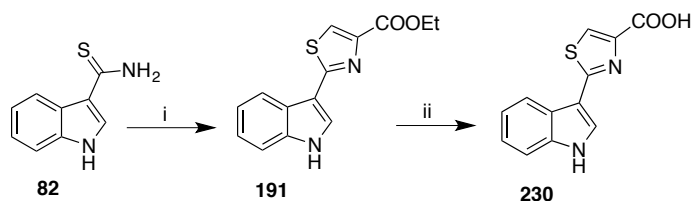
Scheme 3.10 Syntheses of (ethyl 2-(1H-indol-3-yl)thiazole-4-carboxylate (**191**) and 2-(1H-indol-3-yl)thiazole-4-carboxylic acid (**230**). Reagents and conditions: (i) Ethyl bromopyruvate, 95% EtOH, 1 h, reflux, 90%; (ii) NaOH, THF, 14 h, r.t., 89% (Moody et al., 1997).

A solution of indole-3-thiocarboxamide (**82**, 50 mg, 0.30 mmol) and ethyl bromopyruvate (77 mg, 0.40 mmol) in EtOH (95%, 5 ml) was refluxed for 1 h. EtOH was removed using a rotary evaporator to afford (ethyl 2-(1H-indol-3-yl)thiazole-4-carboxylate (**191**, 70 mg, 0.26 mmol, 90%) (Moody et al., 1997).

Melting point: 245 °C (decomposed)

HPLC t_R = 18.7 min (method C).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 220, 275, 325.

FTIR (KBr) ν_{max} cm^{-1}: 3247, 1710, 1540, 1460, 1327, 1227, 908, 725.

^{1}H NMR (500 MHz, CDCl$_3$): δ 8.2 (d, J = 5.5 Hz, 1H), 8.1 (br, 1H), 8.1 (s, 1H), 7.5 (d, J = 7 Hz, 1H), 7.3 (m, 2H), 4.5 (q, J = 6 Hz, 2H), 1.4 (t, J = 6 Hz, 3H).

^{13}C NMR (125.8MHz, CDCl$_3$): δ 164.3, 161.8, 146.5, 136.6, 126.7, 124.7, 124.6, 123.4, 121.8, 120.2, 112.3, 111.2, 61.8, 14.6.

HRMS-FD *m/z*: measured 272.06263 ([M]$^+$, calcd 272.06195 for C$_{11}$H$_8$N$_2$OS) (100%).

Sodium hydroxide (0.30 ml) was added to a solution of (ethyl 2-(1H-indol-3-yl)thiazole-4-carboxylate (**191**, 10 mg, 0.04 mmol) in THF (1 ml). The reaction mixture was stirred at room temperature for 14 h and then was diluted with water and acidified to pH ~ 3 (HCl, 0.50 M). The reaction mixture was extracted with EtOAc. The combined extracts were dried and concentrated to dryness to yield 2-(1H-indol-3-yl)thiazole-4-carboxylic acid (**230**) (8 mg, 0.03 mmol, 89%).

Melting point: 131–132 °C

HPLC t_R = 4.7 min (method C).

UV (HPLC, CH_3OH-H_2O) λ_{max} (nm): 220, 270, 320.

FTIR (KBr) ν_{max} cm^{-1}: 3350, 1695, 1542, 1244, 732.

^{1}H NMR (500 MHz, MeOD): δ 8.2 (s, 1H), 8.1 (m, 1H), 8.0 (s, 1H), 7.5 (m, 1H), 7.2 (m, 2H).

^{13}C NMR (125.8MHz, MeOD): δ 166.3, 164.6, 148.2, 138.5, 127.8, 125.9 (d), 124.0, 122.3, 121.1, 113.2, 111.8.

HRMS-FD m/z: measured 244.02984 ([M]$^+$, calcd 244.03065 for $C_{12}H_8N_2O_2S$) (100%).

3.3.2 Brassinin (9)
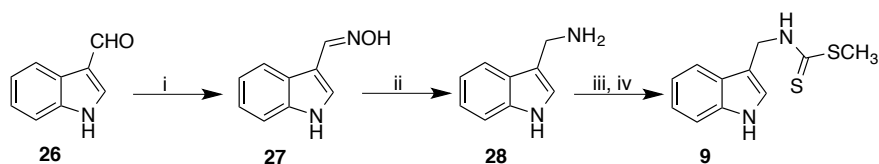
Scheme 3.11 Synthesis of brassinin (**9**). Reagents and conditions: (i) $NH_2OH.HCl$, Na_2CO_3, 1 h, 80 °C, 95%; (ii) $NiCl_2.6H_2O$, $NaBH_4$, MeOH, 10 min, 0 °C, 70%; (iii) Et_3N, pyridine, CS_2, 10 min, 0 °C; (iv) MeI, 30 min, 0 °C, 73% (Sharma-Mamillapalle, 2012).

A solution of $NH_2OH.HCl$ (957 mg, 13.8 mmol) and Na_2CO_3 (803 mg, 7.58 mmol) in water (10 ml) was added to a solution of indolyl-3-carboxaldehyde (**26**, 1.0 g, 7.0 mmol) in EtOH (95%, 25 ml). The mixture was stirred at 80 °C for 1 h then was concentrated. The resulting precipitate was filtered and the precipitate was washed with water to afford indolyl-3-carboxaldoxime (**27**, 1050 mg, 6.600 mmol, 95%) as yellow solid.

$NaBH_4$ (1075 mg, 28.00 mmol) was added portionwise to a solution of indolyl-3-carboxaldoxime (**27**, 700 mg, 4.40 mmol) and $NiCl_2.6H_2O$ (1039 mg, 4.400 mmol) in MeOH (10 ml) at 0 °C followed by stirring at the same temperature for 10 min. The reaction mixture was diluted with mixture of water and NH_4OH (2:1, 50 ml), filtered and extracted with chloroform. The combined extracts were dried and concentrated to dryness. The residue was subjected to FCC (silica gel, $CHCl_3$-MeOH-NH_4OH, 80:20:1.0) to afford 3-indolylmethanamine (**28**, 450 mg,

3.10 mmol, 70%) as white solid (Kutschy et al., 1998).

Carbon disulfide (199 ml, 3.30 mmol) was added to a solution of amine (**28**, 440 mg, 3.01 mmol) and Et₃N (837 ml, 6.0 mmol) in pyridine (3 ml) at 0° C. The mixture was stirred for 10 min at the same temperature followed by addition of methyl iodide (282 ml, 4.50 mmol) and stirring at room temperature for additional 30 min. The reaction mixture was diluted with water and extracted with EtOAc. The combined extracts were dried and concentrated to dryness. The residue was subjected to FCC (silica gel, EtOAc-hexane, 2:8) to afford brassinin (**9**, 521 mg, 2.20 mmol, 73%) as an off-white solid.

Melting point: 132–133 °C

HPLC t_R = 15.3 min (method C).

UV (HPLC, CH_3OH-H_2O) λ_{max}(nm): 217, 270.

^{1}H NMR (500 MHz, CD_3CN): δ 9.25 (br, 1H, D_2O exchangeable), 8.24 (s, 1H, D_2O exchangeable), 7.63 (d, J = 8 Hz, 1H), 7.43 (d, J = 8 Hz, 1H), 7.31 (d, J = 2 Hz, 1H), 7.16 (dd, J = 8, 8 Hz, 1H), 7.08 (dd, J = 8, 8 Hz, 1H), 5.04 (d, J = 5 Hz, 2H), 2.55 (s, 3H) and minor signals 4.77 (d) and 2.64 (s) (ca. 1/10 intensity of the major peaks) due to a rotamer.

3.3.3 Cyclobrassinins

3.3.3.1 *Cyclobrassinin (4)*
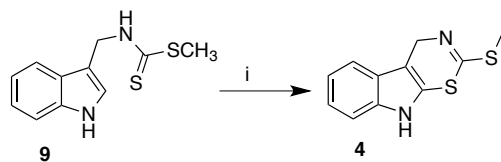
Scheme 3.12 Synthesis of cyclobrassinin (**4**). Reagents and conditions: (i) PBP, DBU, THF, 100 min, r.t., 58% (Takasugi et al., 1986; Pedras et al., 2011b).

Pyridinium bromide perbromide (PBP) (220 mg, 0.60 mmol) was added to a solution of brassinin (**9**, 160 mg, 0.600 mmol) in THF (4 ml). The reaction mixture was stirred at room temperature for 40 min followed by addition of 1,8-diazabicycloundec-7-ene (DBU) (320 μl, 0.320 mmol). The reaction mixture was kept stirring at room temperature for additional 1 h then was concentrated to dryness. The residue was subjected to FCC (silica gel, CH$_2$Cl$_2$-hexanes, 6:4) to afford cyclobrassinin (**4**, 90 mg, 0.40 mmol, 58%) as light yellow solid (Takasugi et al., 1986; Pedras et al., 2011b).

Melting point: 136–137 °C

HPLC t_R = 13.9 min (method B).

UV (HPLC, CH$_3$OH-H$_2$O) λ$_{max}$(nm): 210, 280.

^{1}H NMR (500 MHz, CDCl$_3$): δ 7.9 (br, 1H), 7.5 (d, J = 6.5 Hz, 1H), 7.3 (d, J = 6.5 Hz, 1H), 7.2 (dd, J = 6, 6.5 Hz, 1H), 7.2 (dd, J = 6, 6 Hz, 1H), 5.1 (s, 2H), 2.6 (s, 3H).

3.3.3.2   1-Methylcyclobrassinin (193)
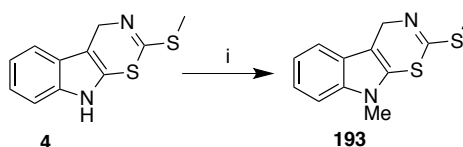
Scheme 3.13 Synthesis of 1-methylcyclobrassinin (**193**). Reagents and conditions: (i) NaH, MeI, THF, 1 h, 0 ºC, 90%.

Sodium hydride 60% suspension in mineral oil (18 mg, 0.40 mmol, washed with hexane) was added to a solution of cyclobrassinin (**4**, 54 mg, 0.20 mmol) in THF (2 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 30 min followed by dropwise addition of methyl iodide (30 μl, 0.48 mmol). The reaction mixture was kept stirring at room temperature for additional 2.5 h then was diluted with water and extracted with EtOAc. The combined extracts were dried and concentrated to dryness. The residue was subjected to FCC (silica gel, EtOAc-hexanes, 1:9) to afford 1-methylcyclobrassinin (**193**, 56 mg, 90%).

Melting point: 95–98 °C

HPLC t_R = 19.1 min (method B).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max}(nm): 206, 230, 300.

^{1}H NMR (500 MHz, CDCl$_3$): δ 7.5 (d, J = 8 Hz, 1H), 7.3 (d, J = 8 Hz, 1H), 7.2 (dd, J = 7.5, 7.5 Hz, 1H), 7.1 (dd, J = 7, 7.5 Hz, 1H), 5.1 (s, 2H), 3.7 (s, 3H), 2.6 (s, 3H).

3.3.4 Rutalexin (5)
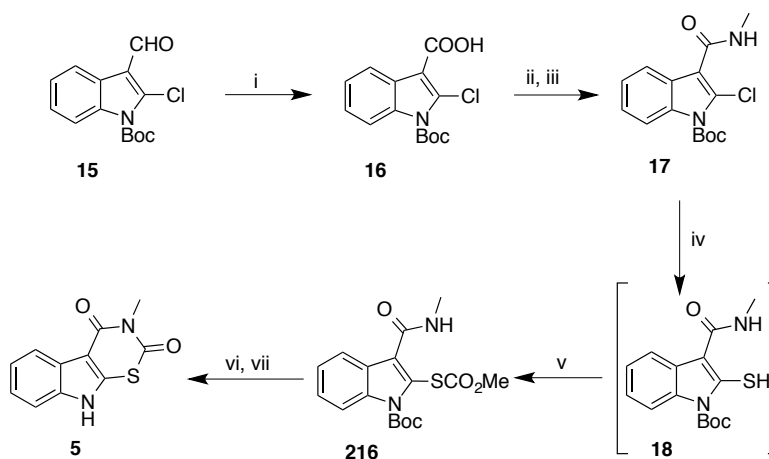
Scheme 3.14 Synthesis of rutalexin (**5**). Reagents and conditions: (i) NaClO$_2$, KH$_2$PO$_4$, 2-methylbut-2-ene, *tert*-butyl alcohol, H$_2$O, 4 h, r.t., 100%; (ii) SOCl$_2$, THF, 2 h, rt.; iii) CH$_3$NH$_2$, THF, 0 °C, 1 h, 95% (over steps **ii** and **iii**); (iv) NaSH, DMF, H$_2$O, 1 h, 0 °C; (v) Methyl chloroformate, 30 min, r.t., 95%; (vi) THF, Et$_3$N, 4 h, r.t.; (vii) TFA (20%) in DCM, 4 h, r.t., 93% (over steps **vi** and **vii**).

A solution of NaClO$_2$ (1 g, 6.80 mmol) and KH$_2$PO$_4$ (1.2 g, 8.8 mmol) in water (6 ml) was added to a mixture of 1-Boc-2-chloroindole-3-carboxaldehyde (**15**, 255 mg, 0.900 mmol), *tert*-butyl alcohol (6.4 ml) and 2-methylbut-2-ene (6.4 ml). The reaction mixture was stirred at room temperature for 4 h. The organic phase was separated and the aqueous layer was acidified (HCl (0.50 M), PH ~ 3) and extracted with EtOAc. The combined extracts were dried and concentrated to dryness to afford 1-Boc-2-chloroindole-3-carboxylic acid (**16**, 270 mg, 0.900 mmol, 100%) (Pedras et al., 2004b).

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Thionyl chloride (250 μl, 3.75 mmol) was added to a solution of acid **16** (90 mg, 0.30 mmol) in dry THF (3 ml) at 0 °C followed by catalytic amount of DMF (5 μl). The reaction mixture was stirred at room temperature for 2 h. The excess amount of thionyl chloride was evaporated using rotary evaporator and the reaction mixture was cooled to 0 °C. A solution of MeNH$_2$ in THF (2 M, 4 ml, 8 mmol) was added slowly (in 10 min) to the reaction mixture and the reaction mixture was stirred for additional 1 h at 0 °C. The reaction mixture was diluted with water and extracted with EtOAc. The combined extracts were dried and concentrated to dryness. The residue was subjected to FCC (silica gel, EtOAc-hexane, 1:1) to afford *tert*-butyl 2-chloro-3-(methylcarbamoyl)-1H-indole-1-carboxylate (**17**, 88 mg, 0.29 mmol, 95%) as colorless oil (Pedras et al., 2004b).

A solution of sodium hydrogen sulfide (360 mg, 3.20 mmol) in water (100 μl) was added to a mixture of *tert*-butyl 2-chloro-3-(methylcarbamoyl)-1H-indole-1-carboxylate (**17**, 50 mg, 0.25 mmol) in DMF (1.5 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h. Methyl chloroformate (1.2 ml, 14 mmol) was added dropwise to the reaction mixture. The ice bath was removed and the reaction mixture was stirred at room temperature for additional 30 min. The reaction mixture was diluted with water, extracted with EtOAc. The combined extracts were dried and concentrated to dryness to afford **216** (56 mg, 0.15 mmol, 95 %) as colorless oil.

HPLC t_R = 11.6 min (method B).
UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 210, 280.
^{1}H NMR (500 MHz, CD$_3$OD): δ 8.10 (d, *J* = 8.5Hz, 1H), 7.98 (d, *J* = 8Hz, 1H), 7.41 (dd, *J* = 7.5, 8Hz, 1H), 7.31 (dd, *J* = 7.5, 7.5 Hz, 1H), 6.5 (br, 1H), 3.9 (s, 3H), 3.0 (d, *J* = 5Hz, 3H), 1.7 (s, 9H).
^{13}C NMR (500 MHz, CD$_3$OD): δ 168.4, 164.2, 149.3, 137.6, 126.8, 126.8, 126.8, 123.9, 121.5, 121.4, 115.5, 85.7, 55.4, 28.2, 26.7.
HRMS-EI *m/z*: measured 364.11078 ([M]$^+$, calcd. 364.10929 for C$_{24}$H$_{28}$N$_2$O$_5$S).

Triethylamine (100 μl) was added to a solution of amide **216** in THF (1 ml) and the reaction mixture was stirred at room temperature for 4 h. After all **216** was consumed, a mixture of 20% TFA in DCM was added to the reaction. The reaction mixture was kept stirring for additional 4 h at room temperature. The reaction mixture was concentrated to dryness and the

residue was washed with diethyl ether (2 ml × 2) to afford pure rutalexin (**5**, 30 mg, 0.13 mmol, 93%) as white powder.

Melting point: 310–312 °C

HPLC t_R = 8.5 min (method B).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 213, 242, 275.

^{1}H NMR (500 MHz, DMSO-d_6): δ 12.57 (br, 1H, D$_2$O exchangeable), 8.09 (m, 1H), 7.54 (m, 1H), 7.28 (m, 2H), 3.37 (s, 3H).

^{13}C NMR (125 MHz, DMSO-d_6): δ 162.8, 160.0, 136.9, 135.0, 125.1, 123.6, 122.1, 119.5, 111.8, 101.5, 28.2.
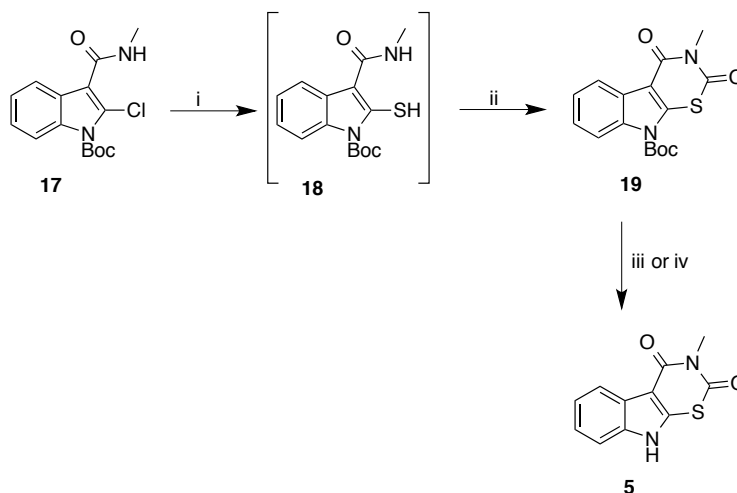
Scheme 3.15   Synthesis of rutalexin (**5**). Reagents and conditions: (i) NaSH, DMF, H$_2$O, 0 °C, 1 h; (ii) Methyl chloroformate, 30 min, **216** (95%) and 6 h, **19** (56%); (iii) TFA (20%) in DCM, r.t., 4 h; (iv) K$_2$CO$_3$, MeOH, r.t., 2 h, 60%.

A solution of sodium hydrogen sulfide (36 mg, 0.50 mmol) in water (50 μl) was added to a mixture of *tert*-butyl 2-chloro-3-(methylcarbamoyl)-1H-indole-1-carboxylate (**17**, 10 mg, 0.050 mmol) in DMF (0.50 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h followed by dropwise addition of methyl chloroformate (50 μl, 0.60 mmol). The ice bath was removed and the reaction mixture was kept stirring at room temperature for an additional 6 h. The reaction mixture was diluted with water and extracted with EtOAc. The combined extracts were

concentrated to dryness. The residue was subjected to FCC (silica gel, EtOAc–hexane, 3:7) to afford *N*-Boc-rutalexin (**19**, 6 mg, 56%) as white powder.

HPLC t_R = 21.3 min (method B).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 216, 253.

^{1}H NMR (500 MHz, CD$_3$CN): δ 8.30 (d, *J* = 6.5, 1H), 8.11 (d, *J* = 7.5, 1H), 7.42 (m, 2H), 3.4 (s, 3H), 1.7 (s, 9H).

iii. TFA (400 μl) was added to a solution of *N*-Boc-rutalexin (**19**, 20 mg, 0.060 mmol) in DCM (2 ml) at room temperature. The reaction mixture was stirred at room temperature for 4 h, followed by concentration to dryness. The residue was rinsed with diethyl ether (2 ml × 2) and dried to afford rutalexin (**5**) in quantitative yield.

iv. Potassium carbonate (5 mg, 0.04 mmol) was added to a solution of *N*-Boc-rutalexin (**19**, 5 mg, 0.02 mmol) in MeOH (1 ml). The reaction mixture was stirred for 2 h at room temperature, followed by concentration to dryness. The residue was subjected to FCC (silica gel, EtOAc–hexane, 3:7) to afford rutalexin (**5**, 2 mg, 60%) as white powder.

HPLC t_R = 8.5 min (method B).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 213, 242, 275.

^{1}H NMR (500 MHz, DMSO-d_6): δ 12.57 (br, 1H, D$_2$O exchangeable), 8.09 (m, 1H), 7.54 (m, 1H), 7.28 (m, 2H), 3.37 (s, 3H).

^{13}C NMR (125 MHz, DMSO-d_6): δ 162.8, 160.0, 136.9, 135.0, 125.1, 123.6, 122.1, 119.5, 111.8, 101.5, 28.2.

3.3.5   Brassilexins

3.3.5.1   *Brassilexin (7)*
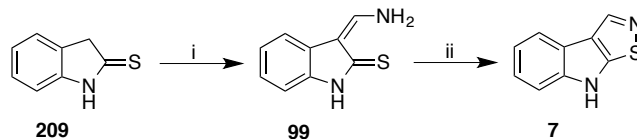
Scheme 3.16 Synthesis of brassilexin (**7**). Reagents and conditions: (i) POCl$_3$/DMF, NH$_4$OH, 20 min, 45 °C then 0 °C; (ii) Pyridine, I$_2$, 1 h, r.t., 51%.

Indole-2-thione (**209**) was added to a solution of freshly distilled POCl$_3$ (64 μl, 0.70 mmol) in DMF (350 μl) at 45 °C. The reaction mixture was stirred for 20 min at 45 °C then was cold to 0 °C on an ice bath. NH$_4$OH (8 ml) was added dropwise to the reaction mixture with constant stirring. The reaction mixture was warmed up to room temperature and was extracted with diethyl ether. The combined extracts were dried and concentrated to dryness. The residue was dissolved in pyridine (1 ml) followed by addition of I$_2$ (42 mg, 0.33 mmol). The reaction mixture was stirred for 1 h at room temperature and then was acidified using H$_2$SO$_4$ (1.5 M, 10 ml) and extracted with diethyl ether. The combined extracts were dried and concentrated to dryness. The residue was subjected to FCC (silica gel, EtOAc-hexane, 2:8) to afford brassilexin (**7**, 30 mg, 0.17 mmol, 51%) (Pedras and Jha, 2005).

Melting point: 140–142 °C

HPLC t_R = 12.6 min (method C).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 220, 250, 265.

^{1}H NMR (500 MHz, CDCl$_3$): δ 8.7 (s, 2H), 7.9 (d, *J* = 6.5 Hz, 1H), 7.5 (d, *J* = 7 Hz, 1H), 7.4 (dd, *J* = 6.5, 6.5 Hz, 1H), 7.3 (dd, *J* = 6.5, 7.5 Hz, 1H).

3.3.5.2 1-Methylbrassilexin (165)
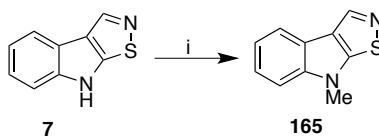
Scheme 3.17 Synthesis of 1-methylbrassilexin (**165**). Reagents and condition: (i) NaH, MeI, THF, 0 ºC, 1 h, 94%.

Sodium hydride 60% suspension in mineral oil (35 mg, 1.5 mmol, washed with hexane) was added to a solution of brassilexin (**24**) (51 mg, 0.29 mmol) in THF (3 ml) at 0 ºC. The reaction mixture was stirred for 15 minutes at 0 ºC followed by addition of methyl iodide (27 μl, 0.44 mmol). The reaction mixture was stirred for an additional 1 h at 0 ºC. Ice-cold water was added to quench the reaction and then the aqueous was extracted with DCM. The combined extracts were dried and concentrated to dryness. The crude reaction mixture was subjected to FCC (silica gel, DCM-hexane, 80:20) to afford 1-methylbrassilexin (**165**, 52 mg, 0.30mmol, 94%) (Pedras and Suchy, 2005).

Melting point: 67–69 °C

HPLC t_R = 15.7 min (method B).

UV (HPLC, CH$_3$OH-H$_2$O) λ$_{max}$ (nm): 220, 250, 270.

^{1}H NMR (500 MHz, CDCl$_3$): δ 8.7 (s, 1H), 7.9 (d, J = 6.5 Hz, 1H), 7.4 (m, 2H), 7.3 (dd, J = 6, 7 Hz, 1H), 3.9 (s, 3H).

3.4 Biotransformation of phytoalexins and related structures by *Alternaria brassicicola*

3.4.1 Camalexins and related structures

3.4.1.1 Biotransformation of camalexins

Time course experiments for camalexins **1**, **2** and **3** were carried out in MM. Fungal cultures of *A. brassicicola* were initiated by inoculating *A. brassicicola* spores (10^6/100 ml) in

minimal media (50 ml in 125 ml Erlenmeyer flask) at 23 ± 1 °C, under constant light, on a shaker at 110 rpm. After 48 h of incubation, compounds to be tested (dissolved in CH_3CN) were added to each culture (triplicate) and uninoculated MM (final concentration in media 0.050 mM). Control cultures of the fungus were grown separately. Samples (5 ml) were withdrawn from the cultures immediately after addition of each compound and then after different time intervals up to 120 h (every 24 h). Samples were extracted with EtOAc (10 ml × 2); the aqueous phase was acidified (HCl) and reextracted with EtOAc (10 ml × 2); the remaining aqueous phase was basified (NaOH) and reextracted with $CHCl_3$ (10 ml × 2). The combined extracts (neutral, acidic and basic separately) were concentrated and residues were dissolved in CH_3CN (0.20 ml) and analyzed by HPLC-DAD-ESI-MS. Transformations of metabolites were carried out similarly. For time course experiments carried out in H_2O, mycelia of cultures were filtered off after 48 h of incubation, were washed with water and transferred into sterile H_2O; a solution of the compound to be tested (dissolved in CH_3CN) was added to the cultures (final concentration of 0.050 mM). Cultures were incubated in a shaker at room temperature and treated as reported above. The EtOAc extract or the freeze-dried residue was dissolved in CH_3CN or MeOH–H_2O (1:1) and analyzed by HPLC-DAD-ESI-MS.

3.4.1.2 *Biotransformation of related structures*

Time course experiments for related structures **173**, **169**, **170**, **168**, **167**, **172**, **171** were carried out in MM and only in case of compound **174** the experiment was carried out in water using the procedure described in **Section 3.4.1.1**.

Metabolite **185** resulted from transformation of compound **173** by *A. brassicicola* was isolated from the larger scale cultures. Cultures (500 ml) were prepared in 250 ml Erlenmeyer flasks containing 100 ml MM. After 48 h of incubation, 4′-methylcamalexin (**173**, 5.5 mg, dissolved in CH_3CN) were added to fungal cultures (final concentration in media 0.050 mM). The cultures were incubated for additional 96 h (at 23 ± 1 °C, under constant light). The cultures were filtered and the mycelia were washed with water. The filtrates (total ca. 500 ml) were combined and concentrated using a freeze-dryer. The residue was extracted with EtOAc (3 × 100 ml). The combined extracts were dried and concentrated under reduced pressure to yield an oily residue (50 mg). The residue was subjected to FCC (silica gel, EtOAc) to yield metabolite **185** (3.00 mg).

3.4.1.3 Synthesis of metabolites

3.4.1.3.1 Indole-3-thiocarboxamide (82) and 1-Methylindole-3-thiocarboxamide (90)
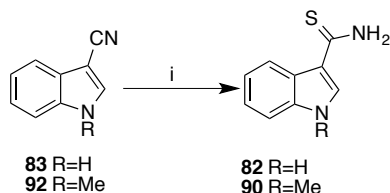
Scheme 3.18 Syntheses of indole-3-thiocarboxamide (**82**) and 1-methylindole-3-thiocarboxamide (**90**). Reagents and conditions: (i) CH_3CSNH_2, 10% HCl/DMF, 12 h, 90 °C, 45% (**82**), 80% (**90**) (Gu et al., 1999).

A mixture of indole-3-carbonitrile (**83**, 100 mg, 0.70 mmol) and thioacetamide (105 mg. 1.40 mmol) in 10% HCl-DMF solution (1.50 ml) was stirred at 90 °C for 12 h. The reaction mixture was then neutralized with $NaHCO_3$ (satd. sol.) and extracted with EtOAc. The combined extracts were dried and concentrated to dryness. The residue was subjected to FCC (silica gel, DCM-MeOH, 99:1) to afford indole-3-thiocarboxamide (**82**, 53 mg, 45%) as yellow powder. 1-Methylindole-3-thiocarboxamide (**90**, 82 mg, 80%, as a light yellow powder) was prepared similarly from 1-methylindole-3-carbonitrile (**92**, 100 mg, 0.70 mmol) (Gu et al., 1999).

Indole-3-thiocarboxamide (82)

Melting point: 151–152 °C

HPLC t_R = 4.6 min (method A).

UV (HPLC, CH_3OH-H_2O) λ_{max} (nm): 215, 255, 318.

FTIR (KBr, cm-1) ν_{max}: 3190, 1621, 1527, 1442, 850.

^{1}H NMR (DMSO-d_6) δ: 10.91 (s, 1H), 8.08 (s, 1H), 7.95 (s, 1H), 7.75 (d, J= 7.5 Hz, 1H), 7.22 (d, J= 3.0 Hz, 1H), 6.56 (d, J= 8.0 Hz, 1H), 6.29 (dd, J= 7.0, 7.0 Hz, 1H), 6.26 (dd, J= 7.0, 7.0 Hz, 1H).

^{13}C NMR (DMSO-d_6) δ: 193.6, 136.8, 128.1, 125.9, 122.0, 121.8, 120.7, 116.3, 112.0.

HRMS-EI m/z: measured 176.0409 ([M]$^+$, calcd. 176.0408 for $C_9H_8N_2S$) (100%).

1-Methylindole-3-thiocarboxamide (90)

Melting point: 125–128 °C.

HPLC t_R = 6.6 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 220, 260, 330.

^{1}H NMR (500 MHz, CD$_3$CN): δ 8.32 (d, J= 7.5 Hz, 1H), 8.93 (s, 1H), 7.65 (br, 2H), 7.43 (d, J= 7.5 Hz, 1H), 7.27 (m, 2H), 3.80 (s, 3H).

^{13}C NMR (125.8MHz, CD$_3$CN): δ 195.8, 138.8, 135.3, 126.2, 123.6, 122.7, 122.1, 116.8, 111.6, 33.99.

3.4.1.3.2   *6-Methoxyindole-3-carbonitrile (93) and 6-methoxyindole-3-thiocarboxamide (91)*
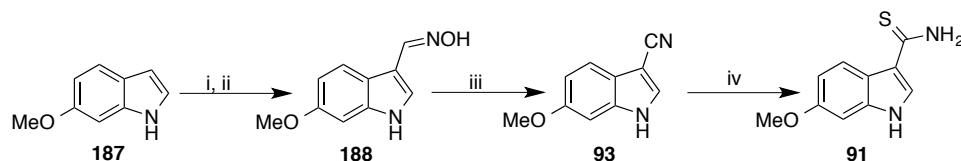
Scheme 3.19 Syntheses of 6-methoxyindole-3-carbonitrile (**93**) and 6-methoxyindole-3-thiocarboxamide (**91**). Reagents and conditions: (i) POCl$_3$, DMF, 2.5 h, 98%; (ii) HONH$_2$, HCl, NaOAc, 95%, EtOH, H$_2$O, 3 h, r.t.; (iii) Ac$_2$O, pyridine, THF, reflux, 9 h. 96%; (iv) CH$_3$CSNH$_2$, HCl/DMF, 12 h, 90 °C, 40%.

A solution of 6-methoxyindole (**187**) in DMF (0.04 ml) was added to an ice-cold mixture of DMF (0.07 ml) and POCl$_3$ (0.07 ml) and the reaction mixture was stirred at room temperature for 2.5 h. The reaction mixture was diluted with water and extracted with EtOAc. The combined extracts were dried and concentrated to dryness to yield 6-methoxyindol-3-carbaldehyde in 98% yield. A solution of HONH$_2$.HCl (27 mg, 0.40 mmol), NaOAc (31 mg, 0.38 mmol) and H$_2$O (50 μl) was added to a solution of 6-methoxyindol-3-carbaldehyde (50 mg, 0.30 mmol) in EtOH (95%, 4 ml). The reaction mixture was stirred at room temperature for 3 h, EtOH was removed and the residue was diluted with H$_2$O and extracted with EtOAc. The combined extracts were dried and concentrated to dryness to yield 6-methoxyindol-3-oxime (**188**). Pyridine was added to

a solution of 6-methoxyindol-3-oxime (**188**, 50 mg, 0.25 mmol) in THF (3 ml) and Ac$_2$O (50 μl, 0.50 mmol) under argon and the reaction mixture was refluxed for 9 h. Pyridine was removed in a rotary evaporator using toluene to afford 6-methoxyindole-3-carbonitrile (**93**, 43 mg, 96%) as a light yellow powder.

Melting point: 180 °C (decomposed; lit 174 °C, decomposed).
HPLC t_R = 7.9 min (method A).
UV (HPLC, CH$_3$OH-H$_2$O) λ$_{max}$ (nm): 220, 270, 290.
FTIR (KBr) ν$_{max}$ cm^{-1}: 3383, 1612, 1451, 1122, 739.
^{1}H NMR (500 MHz, CD$_3$CN): δ 8.50 (br, 1H), 7.60 (s, 1H), 7.63 (m, 2H), 6.97 (dd, J= 8.5, 2 Hz 1H), 6.93 (s, 1H), 3.91 (s, 3H).
^{13}C NMR (500MHz, CD$_3$CN): d 157.6, 136.2, 132.4, 120.8, 119.4, 115.9, 112.2, 95.4, 85.4, 55.2.
HRMS-EI m/z: measured 172.0638 ([M]$^+$, calcd. 172.0637 for C$_{10}$H$_8$N$_2$O) (100%).

A solution of 6-methoxyindole-3-carbonitrile (**93**, 50 mg, 0.50 mmol) and thioacetamide (55 mg, 0.70 mmol) in 10% HCl-DMF (1 ml) was stirred at 90 °C for 12 h. The reaction mixture was neutralized with NaHCO$_3$ (satd. sol.) and then was extracted with EtOAc. The combined extracts were dried, concentrated to dryness and the residue was subjected to FCC (silica gel, MeOH–DCM, 0.30:9.7) to afford 6-methoxyindole-3-thiocarboxamide (**91**, 24 mg, 40%) as a light brownish powder.

Melting point: 133–135 °C.
HPLC t_R = 4.5 min (method A).
UV (HPLC, CH$_3$OH-H$_2$O) λ$_{max}$ (nm): 220, 250, 280.
FTIR (KBr) ν$_{max}$ cm^{-1}: 3399, 3250, 1630, 1530, 1040.
^{1}H NMR (500 MHz, CD$_3$CN): δ 9.68 (br, 1H), 8.24 (d, J= 8.5 Hz, 1H), 7.83 (d, J= 2.5 Hz, 1H), 7.60 (br, 2H), 7.00 (d, J= 2.5 Hz, 1H), 6.86 (dd, J= 8.5, 2.5 Hz, 1H), 3.81 (s, 3H).
^{13}C NMR (500 MHz, CD$_3$CN): d 195.3, 156.7, 137.9, 128.0, 121.9, 119.25, 111.4, 100.0, 95.1, 55.1.
HRMS-EI m/z: measured 206.0515 ([M]$^+$, calcd. 206.0514 for C$_{10}$H$_{10}$N$_2$OS) (100%).

3.4.1.3.3   1-Methylindole-3-carbonitrile (92)
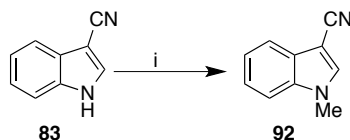
Scheme 3.20 Synthesis of 1-methylindole-3-carbonitrile (**92**). Reagents and conditions: (i) NaH, MeI, THF, 2 h, 0 °C, quantitative (Pedras and Liu, 2004).

Sodium hydride 60% suspension in mineral oil (19 mg, 0.48 mmol, washed with hexane) was added to a solution of indole-3-carbonitrile (**83**, 33 mg, 0.23 mmol) in THF (4 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 10 min followed by dropwise addition of methyl iodide (0.020 ml, 0.32 mmol). The reaction mixture was kept stirring at 0 °C for an additional 2 h. The reaction mixture was diluted with water and extracted with EtOAc. The combined extracts were dried and concentrated to dryness to yield 1-methylindole-3-carbonitrile (**92**, 36 mg, 0.2 mmol, quantitative) (Pedras and Liu, 2004).

Melting point: 53–55 °C

HPLC t_R = 14.6 min (method A).

UV (HPLC, CH_3OH-H_2O) λ_{max} (nm): 220, 280.

FTIR: 3331, 3116, 2932, 2223, 1528, 1459, 1383, 1335, 1254, 1195, 1136 cm^{-1}.

^{1}H NMR (500 MHz, CD_3OD): δ 7.91 (s, 1H), 7.72 (d, J= 8 Hz, 1H), 7.55 (d, J= 8 Hz, 1H), 7.37 (dd, J= 8, 7.5 Hz, 1H), 7.29 (dd, J= 8, 7.5 Hz, 1H), 3.89 (s, 3H).

^{13}C NMR (500 MHz, CD_3CN): δ 135.5, 135.2, 126.5, 122.3, 120.6, 114.5, 114.5, 109.4, 82.7, 31.2.

HRMS-EI m/z: measured 156.0687 ([M]$^+$, calcd. 156.0687 for $C_{10}H_8N_2$) (100%).

*3.4.1.3.4   1-Methylindole-3-carboxylic acid (94) and 6-methoxyindole-3-carboxylic acid
(95)*

189 R$_1$=Me, R$_2$=H
190 R$_1$=H, R$_2$=OMe

94 R$_1$=Me, R$_2$=H
95 R$_1$=H, R$_2$=OMe
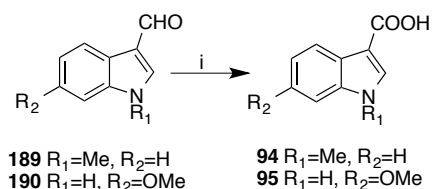
Scheme 3.21 Syntheses of 1-methylindole-3-carboxylic acid (**94**) and 6-Methoxyindole-3-carboxylic acid (**95**). Reagents and conditions: (i) NaClO$_2$, NaH$_2$PO$_4$, *t*-butanol, 2-methylbut-2-ene, H$_2$O, r.t., 14 h, 75% (**94**) and 60 h, 52% (**95**).

A solution of NaClO$_2$ (25 eq) and NaH$_2$PO$_4$ (20 eq) in water (1 ml) was added to a mixture of 1-methylindole-3-carbaldehyde (**189**, 25 mg, 0.15 mmol) in *t*-butanol (1.5 ml) and 2-methylbut-2-ene (1.5 ml). The reaction mixture was stirred at room temperature for 14 h (**94**) followed by dilution with water and extraction with EtOAc. The combined extracts were dried and concentrated to dryness. The residue was subjected to FCC (silica gel, MeOH-DCM, 1:9) to afford 1-methylindole-3-carboxylic acid (**94**, 20 mg, 75%) as a light yellow powder.

6-Methoxyindole-3-carboxylic acid (**95**, 10 mg, 52%, as a light yellow powder) was prepared similarly from 6-methoxyindole-3-carboxaldehyde (**190**) after 60 h stirring at the same condition.

1-Methylindole-3-carboxylic acid (94)

Melting point: 200–204 °C

HPLC t_R = 4.7 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 215, 290.

^{1}H NMR (500 MHz, MeOD): δ 8.04 (d, *J*= 8 Hz, 1H), 7.88 (s, 1H), 7.41 (d, *J*= 8 Hz, 1H), 7.23 (dd, *J*= 7 Hz, 1H), 7.18 (dd, *J*= 7 Hz, 1H), 3.84 (s, 3H).

^{13}C NMR (500 MHz, MeOD): δ 167.4, 137.5, 135.8, 126.8, 122.3, 121.2, 120.9, 109.7, 106.3, 32.1.

HRMS-EI *m/z*: measured 175.0638 ([M]$^+$, calcd 172.0633 for C$_{10}$H$_9$N$_1$O$_2$) (100%).

6-Methoxyindole-3-carboxylic acid (95)

Melting point: 193–194 °C

HPLC t_R = 2.9 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 215, 275.

^{1}H NMR (500 MHz, MeOD): δ 9.78 (s, 1H), 7.98 (s, 1H), 7.96 (d, J= 5 Hz, 1H), 6.96 (s, 1H), 6.86 (d, J= 6 Hz, 1H), 3.81 (s, 3H).

^{13}C NMR (500 MHz, MeOD): δ 167.9, 156.8, 137.6, 130.9, 121.2, 120.3, 111.1, 107.3, 94.5, 54.5.

HRMS-EI m/z: measured 1910585 ([M]$^+$, calcd. 191.0582 for C$_{10}$H$_9$N$_1$O$_3$).

3.4.1.3.5 Indole-3-carboxamide (186)
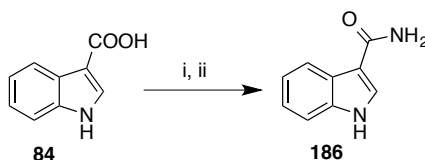
Scheme 3.22 Synthesis of indole-3-carboxamide (**186**). Reagents and conditions: (i) SOCl$_2$, THF, 1 h, 60 °C; (ii) NH$_4$OH, diethyl ether, 3 h, r.t, 80% over 2 steps.

Thionyl chloride (0.30 ml, 7 mmol) was added dropwise to a solution of indole-3-carboxylic acid (20 mg, 0.12 mmol) in freshly distilled THF (0.50 ml). The reaction mixture was heated at 60 °C for 1 h, concentrated to dryness and the residue was used immediately for the next step. The residue was dissolved in dry diethyl ether (1.5 ml) and added dropwise to a cold solution of NH$_4$OH (0.25 ml) in diethyl ether. The reaction mixture was stirred at room temperature for 3 h followed by dilution with water and extraction with EtOAc. The combined extracts were dried and concentrated to dryness. The residue was subjected to FCC (silica gel, EtOAc-hexane, 1:1) to afford indole-3-carboxamide (**186**, 15 mg, 80%) in white powder.

HPLC t_R = 3.7 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 220, 280.

^{1}H NMR (500 MHz, MeOD): δ 8.1 (d, J= 7.5 Hz, 1H), 7.9 (s, 1H), 7.4 (d, J=7.5 Hz, 1H), 7.1 (m, 2H).

^{13}C NMR (500 MHz, MeOD): δ 171.08, 138.3, 130.2, 127.3, 123.6, 122.2, 121.9, 112.9, 111.9.

HRMS-EI m/z: measured 160.0636 ([M]$^+$, calcd. 160.0636 for C$_9$H$_8$N$_2$O) (100%).

3.4.1.3.6 1H-3-(4-Hydroxymethyl-2-thiazolyl)indole (185)
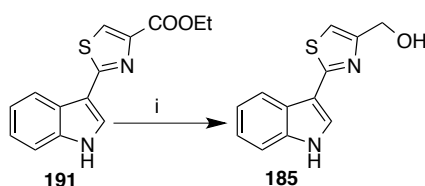
Scheme 3.23 Synthesis of 2–1H-3-(4-hydroxymethyl- 2-thiazolyl)indole (**185**). Reagent and condition: (i) LiAlH$_4$, THF, 10 h, r.t., 83%.

Lithium aluminum hydride (1.5 mg, 0.040 mmol) was added to an ice-cold solution of **191** (10 mg, 0.040 mmol) in dry THF (1 ml) at 0 °C under argon and the reaction mixture was stirred at 0 °C for 10 min. The ice bath was removed and the reaction mixture was stirred at room temperature for an additional 10 h. The reaction mixture was quenched with addition of 25% aq. NaOH (0.50 ml) then filtered and concentrated to dryness. The residue was subjected to FCC (silica gel, EtOAc-hexane, 7:3) to afford (2–1H-3-(4-hydroxymethyl- 2-thiazolyl)indole (**185**, 7 mg, 83%) as a white powder,

Melting point: 196–198 °C.

HPLC t_R = 10.2 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 220, 280, 320.

FTIR (KBr) ν_{max} cm^{-1}: 3191, 1534, 1444, 1241, 1014, 727.

^{1}H NMR (500 MHz, CD$_3$OD): δ 8.1 (d, J= 7 Hz, 1H), 7.9 (s, 1H), 7.4 (d, J= 7 Hz, 1H), 7.2 (m, 3H), 4.7 (s, 2H).

^{13}C NMR (500 MHz, CD$_3$OD): δ 164.6, 156.3, 136.9, 125.2, 124.4, 122.3, 120.5, 119.4, 111.6, 111.1, 110.7, 59.9.

HRMS-EI *m/z*: measured 230.0513 ([M]$^+$, calcd. 230.0514 for C$_{12}$H$_{10}$N$_2$SO) (100%).

3.4.1.3.7 *2-(1H-indol-3-yl)thiazole-5-carboxylic acid (184)*
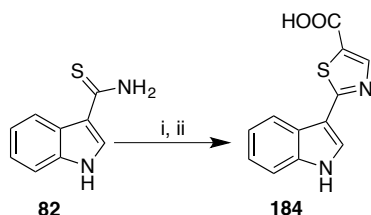
Scheme 3.24 Synthesis of 2-(1*H*-indol-3-yl)-5-thiazolecarboxylic acid (**184**). Reagents and conditions: (i) Methyl 2-chloro-3-oxopropanoate, 95% EtOH, reflux, 5 h, 62%; (ii) NaOH, THF, 4 h, reflux, quantitative.

Methyl 2-chloro-3-oxopropanoate (Gangjee et al., 2001) was added to a solution of indole-3-thiocarboxamide (**82**, 50 mg, 0.28 mmol) in EtOH (95%, 2 ml) and then was refluxed for 5 h. The reaction mixture was concentrated to dryness and the residue was subjected to FCC (silica gel, EtOAc-Hexane, 3:7) to yield methyl 2-(1*H*-indol-3-yl)thiazole-5-carboxylate (45 mg, 62%). NaOH (25% aq, w/v, 0.30 ml) was added to a solution of 2-(1*H*-indol-3-yl)thiazole-5-carboxylate (40 mg, 0.16 mmol) in THF (2 ml) and the reaction mixture was refluxed for 4 h. THF was removed and the residue was diluted with H$_2$O (20 ml) and acidified (pH ≤ 3) with 37% HCl. The aqueous solution was extracted using EtOAc; the combined extracts were dried and concentrated to dryness to afford 2-(1*H*-indol-3-yl)-5-thiazolecarboxylic acid (**230**, 38 mg, quantitative) as a yellow powder.

Melting point: 215 °C (decomposed).

HPLC t_R = 4.5 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ$_{max}$ (nm): 220, 280, 340.

FTIR (KBr) ν$_{max}$ cm^{-1}: 3199, 2392, 1637, 1526, 1220, 1081, 826.

^{1}H NMR (500 MHz, CD$_3$OD): δ 8.29 (s, 1H), 8.11 (m, 1H), 8.07 (s, 1H), 7.49 (m, 1H), 7.26 (m, 2H).

^{13}C NMR (500 MHz, CD$_3$OD): δ 171.3, 164.7, 149.5, 138.6, 128.7, 127.3, 125.8, 124.2, 122.7, 121.2, 113.4, 112.1.

HRMS-EI *m/z*: measured 244.0307 ([M]$^+$, calcd. 244.0306 for C$_{12}$H$_8$N$_2$O$_2$S) (100%).

3.4.2   Cyclobrassinin and 1-methylcyclobrassinin

3.4.2.1   *Biotransformation of cyclobrassinin and 1-methylcyclobrassinin*

Time course experiments for cyclobrassinins **4** and **193** were carried out in MM. Cultures of *A. brassicicola* were initiated by inoculating *A. brassicicola* spores (10^6/100 ml, using diluted solution of spores) in minimal media (5 ml in 25 ml Erlenmeyer flasks) following the procedure described in **Section 3.4.1.1**. Triplicate cultures and controls were prepared separately for each time point. After 48 h of incubation, compounds to be tested (dissolved in CH$_3$CN) were added to the cultures and to uninoculated media (final concentration in media 0.10 mM). Control cultures of fungus were grown separately. Cultures were filtered at different time intervals up to 8 h (0, 2, 4, 8 h) separately, filtrate was extracted with EtOAc (2 × 10 ml) and mycelia were rinsed with EtOAc (5 ml). The combined EtOAc extracts were concentrated to dryness. The residues were dissolved in CH$_3$CN (0.20 ml) and analyzed by HPLC-DAD-ESI-MS. Similarly, time course experiments of compounds **137** and **196** were carried out in H$_2$O. A solution of the compounds to be tested (dissolved in DMSO) was added to the cultures and to uninoculated sterile water (final concentration in media 0.10 mM). Cultures were filtered at different time intervals up to 8 h (0, 2, 4, 8 h) separately and the mycelia were rinsed with water. The combined filtrates were freeze-dried and the residue was dissolved in MeOH-H$_2$O (50:50, 0.20 mM) and analyzed by HPLC-DAD-ESI-MS.

3.4.2.2 Synthesis of metabolites

3.4.2.2.1 Synthesis of compounds 137, 196 and 201-204
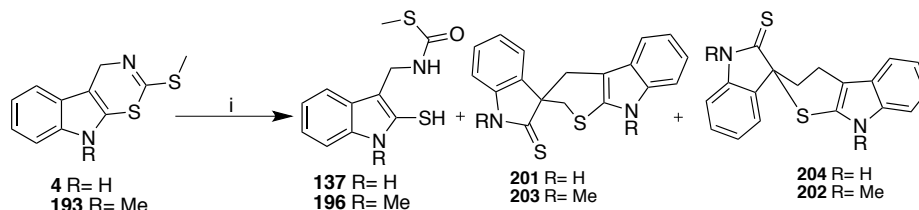
Scheme 3.25 Syntheses of compounds **137**, **196** and **201-204**. Reagent and condition: (i) HCl (0.50 M), 1,4-dioxane, 40 °C.

Hydrochloric acid (0.20 ml, 0.50 M) was added dropwise to a solution of cyclobrassinin (**4**, 20 mg, 0.09 mmol) in 1,4-dioxane (1 ml) and the reaction mixture was kept at 40 °C for 24 h. The reaction mixture was concentrated to dryness and the residue was washed with DCM to yield crude **137** in 51% yield. The DCM solution was concentrated and the residue was subjected to FCC (silica gel, EtOAc-hexane, 30:70) to yield a mixture of **201** and **204** (3 mg, 11%). **204** was the minor isomer in the resultant mixture and the purified amount was not sufficient for fully characterization. Similar reaction was carried out using 1-methylcyclobrassinin (**193**, 20 mg, 0.085 mmol) yielded **196** in 35% yield and a mixture of **203** and **202** in 7% yield.

S-methyl [(2-sulfanyl-1H-indolyl-3)methyl]carbamothioate (137)

Yellow powder.

Melting point: 148–150 °C

HPLC t_R = 15.8 min (method A).

UV (HPLC, CH_3OH-H_2O) λ_{max} (nm): 218, 335(br).

FTIR (KBr) ν_{max} cm^{-1}: 3408, 3319, 2924, 1644, 1621, 1507, 1211.

^{1}H NMR (500 MHz, DMSO-d$_6$): δ 11.57 (br, 1H), 8.22 (brt, *J*= 2.5 Hz, 1H), 7.56 (d, *J*= 7.5 Hz, 1H), 7.37 (d, *J*= 7.5Hz, 1H), 7.22 (dd, *J*= 7.5,7.5 Hz 1H), 7.05 (dd, *J*= 7.5,7.5 Hz, 1H), 3.92 (brd, *J*= 2.5 Hz, 1H), 2.12 (s, 3H).

^{13}C NMR (500 MHz, DMSO-d$_6$): δ 165.8, 137.4, 126.2, 126.1, 123.9, 120.1, 119.8, 119.7, 111.6, 34.4, 11.5.

HRMS-EI *m/z*: measured 250.0231 ([M-2]$^+$, calcd. 250.0234 for C$_{11}$H$_{10}$N$_2$OS$_2$). MS (EI) *m/z* (% relative int.): 250.02 (52), 161.03(100).

S-methyl [(1-methyl-2-sulfanyl-1H-indolyl-3)methyl] carbamothioate (196)

Yellow powder.

Melting point: 157–158 °C.

HPLC t_R = 21.4 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ$_{max}$ (nm): 220, 345.

FTIR (KBr) ν$_{max}$ cm^{-1}: 3318, 2929, 1640, 1524, 1459, 1370, 1308,1217.

^{1}H NMR (500 MHz, DMSO-d$_6$): δ 8.20 (br, 1H), 7.57 (d, *J*= 7 Hz, 1H), 7.53 (d, *J*= 8 Hz, 1H), 7.31 (dd, *J*= 7, 8 Hz 1H), 7.10 (dd, *J*= 7, 8 Hz, 1H), 3.67 (br, 3H), 3.64 (br, 1H), 2.10 (s, 3H). ^{13}C NMR (500 MHz, DMSO-d$_6$): δ 165.6, 138.1, 125.2, 124.3, 123.5, 120.0, 119.9, 115.7, 110.5, 34.5, 29.8, 11.4.

HRMS-EI *m/z*: measured 264.0391 ([M-2]$^+$, calcd. 264.0391 for C$_{12}$H$_{12}$N$_2$OS$_2$). MS (EI) *m/z* (% relative int.): 264.03 (56), 175.04 (100).

4',9'-Dihydro-2'H-spiro[indoline-3,3'-thiopyrano[2,3-b]indole]-2-thione (201)

Brownish powder.

Melting point: 140–143 °C.

HPLC t_R = 17.9 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ$_{max}$ (nm): 238, 308.

FTIR (KBr) ν$_{max}$ cm^{-1}: 3391, 2922, 2852, 1703, 1618, 1496, 1464, 1450, 1348, 1249, 1226, 1140, 1016.

^{1}H NMR (500 MHz, CDCl$_3$): δ 9.51 (s, 1H), 7.94 (s, 1H), 7.36 (d, *J*= 7.5 Hz, 1H), 7.34 (d, *J*= 7.5 Hz, 1H), 7.31 (ddd, *J*= 7.5, 7.5, 1 Hz, 1H), 7.23 (d, *J*= 7.5 Hz 1H), 7.18 (dd, *J*= 7.5, 7.5 Hz, 1H), 7.10 (dd, *J*= 7.5, 7.5 Hz, 1H), 7.08 (d, *J*= 7.5 Hz, 1H), 7.01 (ddd, *J*= 7.5, 7.5, 1 Hz, 1H), 3.87 (d, *J*= 13 Hz, 1H), 3.65 (d, *J*= 16 Hz, 1H), 2.83 (dd, *J*= 16, 2 Hz), 2.64 (dd, *J*= 13, 2 Hz, 1H).

^{13}C NMR (500 MHz, CDCl$_3$): δ 210.8, 141.3, 136.5, 135.7, 128.5, 128.5, 126.7, 124.7, 123.9, 121.5, 119.8, 116.7, 110.1, 109.7, 106.2, 54.7, 37.1, 32.4.

HRMS-EI *m/z*: measured 322.0607 ([M]$^+$, calcd. 322.0598 for C$_{18}$H$_{14}$N$_2$S$_2$). MS (EI) *m/z* (%relative int.): 322.06 [M$^+$] (24), 289.08 (12), 161.03 (100), 117.06 (38).

3.4.2.2.2 Synthesis of compounds 194 and 195
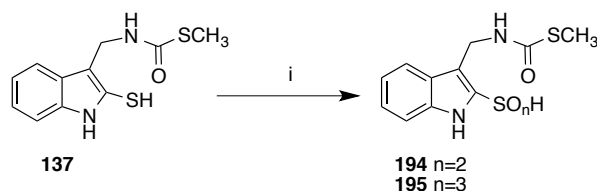
Scheme 3.26 Syntheses of acid **194** and **195**. Reagents and conditions (i) MeOH:CHCl$_3$ (1:1), r.t., H$_2$O$_2$ (16.5eq),16 h, **194** (91%) or H$_2$O$_2$ (32 eq), 20 h, **195**.

Hydrogen peroxide (82 μl, 0.66 mmol) was added to a solution of compound **137** (10 mg, 0.040 mmol) in a mixture of MeOH-CHCl$_3$ (1:1, 2 ml). The reaction mixture was stirred at room temperature for 16 h. The reaction mixture was concentrated to dryness and the residue was washed with MeOH to yield sulfinic acid **194** in 91% yield. Compound **195** was prepared similarly from compound **137** (10 mg, 0.040 mmol), hydrogen peroxide (164 μl) and stirring for 20 h.

Sulfinic acid 194

HPLC-ESI-MS [M-1]$^-$: *m/z* 283

^{1}H NMR (500 MHz, DMSO-d$_6$): δ 11.6 (s, 1H), 8.2 (s, 1H), 7.6 (d, *J*= 6.5 Hz, 1H), 7.4 (d, *J*= 7 Hz, 1H), 7.2 (dd, *J*= 6.5, 6 Hz 1H), 7.1 (dd, *J*= 6, 6.5 Hz, 1H), 3.9 (d, *J*= 3.5 Hz, 1H), 2.1 (s, 3H).

Sulfonic acid 195

HPLC-ESI-MS [M-1]$^-$: *m/z* 299

^{1}H NMR (500 MHz, DMSO-d$_6$): δ 11.6 (s, 1H), 8.2 (s, 1H), 7.6 (d, *J*= 8.5 Hz, 1H), 7.4 (d, *J*= 7.5 Hz, 1H), 7.2 (dd, *J*= 7, 8 Hz 1H), 7.1 (dd, *J*= 8.5, 6.5 Hz, 1H), 3.9 (d, *J*= 3.5 Hz, 1H), 2.1 (s, 3H).

3.4.2.3 Synthesis of compounds 202, 203, 211 and 212
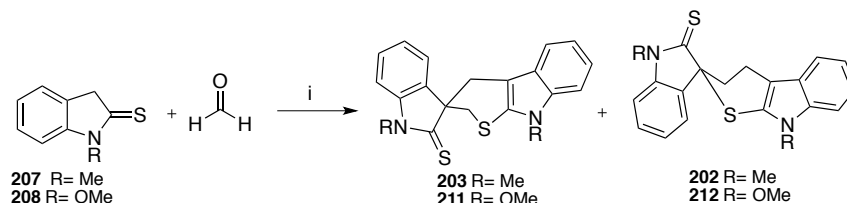
Scheme 3.27 Syntheses of compounds **202**, **203**, **211** and **212**. Reagent and condition: (i) HCl (0.50 ml, 0.50 M) or TFA, MeOH, r.t.

Formaldehyde (540 mg, 6.66 mmol, 37% aq solution, w/w, containing 7-8% MeOH) was added to a stirred solution of **207** or **208** in MeOH (0.50 ml) at room temperature, followed by HCl (0.50 ml, 0.50 M) or TFA (0.10 ml) and the reaction mixture was stirred at room temperature for 16 h. The reaction mixture was diluted with water and the aqueous was extracted with EtOAc. The combined extracts were dried and concentrated to dryness. The residue was subjected to FCC (silica gel, EtOAc-hexane, 1:9) to afford a mixture of **196**, **202**, **211**and **212**. Isomers for each set were separated using FCC (silica gel, EtOAc-hexane, 0.500:99.5). Yield for mixture of **196** and **202** was 32% for HCl; 20% for TFA and for mixture of **211** and **212** was 46% for HCl; 33% for TFA.

1,9'-Dimethyl-4',9'-dihydro-3'H-spiro[indoline-3,2'-thiopyrano[2,3-b]indole]-2-thione (202)

Yellow powder.

Melting point: 155–160 °C.

HPLC t_R = 24.8 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ$_{max}$ (nm): 237, 297, 330.

163

FTIR (KBr) v_{max} cm^{-1}: 3048, 2924, 1720, 1610, 1464, 1369, 1308, 1186, 1162, 1095.

^{1}H NMR (500 MHz, CDCl$_3$): δ 7.51 (d, J= 7.5 Hz, 1H), 7.36 (dd, J= 7.5, 7.5 Hz, 1H), 7.30 (d, J= 7.5 Hz, 1H), 7.21 (dd, J= 7.5, 7.5 Hz, 1H), 7.16 (dd, J= 7.5, 7.5 Hz 1H), 7.11 (d, J= 7.5 Hz, 1H), 7.09 (d, J= 7.5, Hz, 1H), 7.01 (dd, J= 7.5, 7.5, Hz, 1H), 3.74 (s, 3H), 3.59 (s, 3H), 3.33 (ddd, J= 17, 12, 6 Hz, 1H), 3.10 (ddd, J= 17, 12, 6 Hz, 1H), 2.85 (ddd, J= 14, 12, 6 Hz 1H), 2.04 (ddd, J= 14, 12, 6 Hz, 1H).

^{13}C NMR (500 MHz, CDCl$_3$): δ 203.2, 145.5, 137.8, 136.5, 129.1, 127.6, 127.4, 124.9, 124.2, 120.8, 119.3, 117.0, 109.7, 108.4, 104.9, 62.5, 34.8, 32.1, 29.8, 18.5.

HRMS-EI m/z: measured 350.0906 ([M$^+$], calcd. 350.0911 for C$_{20}$H$_{18}$N$_2$S$_2$). MS (EI) m/z (% relative int.): 350.09 [M$^+$] (11), 317.11 (12), 175.04 (100), 130.06 (22).

1,9'-Dimethyl-4',9'-dihydro-2'H-spiro[indoline-3,3'-thiopyrano[2,3-b]indole]-2-thione (203)

Brownish powder.

Melting point: 158–163 °C.

HPLC t_R = 26.0 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 237, 300.

FTIR (KBr) v_{max} cm^{-1}: 3365, 2924, 2852, 1715, 1611, 1570, 1464, 1371, 1182, 1091, 1023.

^{1}H NMR (500 MHz, CDCl$_3$): δ 7.36 (dd, J= 7.5, 7.5 Hz, 1H), 7.32 (d, J= 7.5 Hz, 1H), 7.31 (d, J= 7.5 Hz, 1H), 7.21 (d, J= 7.5 Hz, 1H), 7.18 (dd, J= 7.5, 7.5 Hz 1H), 7.10 (d, J= 7.5 Hz, 1H), 7.06 (dd, J= 7.5, 7.5 Hz, 1H), 7.03 (dd, J= 7.5, 7.5, Hz, 1H), 3.88 (d, J= 13 Hz, 1H), 3.78 (s, 3H), 3.77 (s, 3H), 3.64 (d, J= 16 Hz, 1H), 2.77 (dd, J= 16, 2 Hz), 2.58 (dd, J= 13, 2 Hz, 1H).

^{13}C NMR (500 MHz, CDCl$_3$): δ 208.6, 144.2, 137.5, 135.5, 128.4, 128.1, 127.5, 126.4, 124.1, 120.8, 119.2, 116.7, 109.3, 108.2, 104.8, 54.0, 37.1, 32.9, 32.0, 30.1.

HRMS-EI m/z: measured 350.0911 ([M$^+$], calcd. 350.0911 for C$_{20}$H$_{18}$N$_2$S$_2$). MS (EI) m/z (% relative int.): 350.09 [M+] (34), 175.04 (100).

1,9'-Dimethoxy-4',9'-dihydro-2'H-spiro[indoline-3,3'-thiopyrano[2,3-b]indole]-2-thione (211)

Yellow crystals.

Melting point: 162–165 °C.

HPLC t_R = 25.5 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 235, 305.

FTIR (KBr) ν_{max} cm^{-1}: 2933, 1721, 1617, 1457, 1383, 957.

^{1}H NMR (500 MHz, CDCl$_3$): δ 7.43 (d, J= 10 Hz, 1H), 7.38 (dd, J= 10, 5 Hz, 1H), 7.22 (m, 6H), 7.29 (d, J= 10 Hz, 1H), 4.2 (s, 3H), 4.10 (s, 3H), 3.86 (d, J= 15 Hz, 1H), 3.58 (d, J= 13 Hz, 1H), 2.73 (d, J= 15 Hz, 1H), 2.63 (d, J= 10, 1H).

^{13}C NMR (500 MHz, CDCl$_3$): δ 198.4, 140.3, 133.9, 132.4, 128.7(d), 126.6, 124.9, 121.8, 120.3(d), 116.9, 108.2, 107.2, 102.7, 65, 62.2, 52.5, 36.3, 31.9.

HRMS-EI m/z: measured 382.0812 ([M$^+$], calcd. 382.0800 for C$_{20}$H$_{18}$N$_2$O$_2$S$_2$). MS (EI) m/z (% relative int.): 382.08 (57), 351.06 (100), 319.98 (47), 274.05 (65), 191.03 (29), 161.03 (92).

1,9'-Dimethoxy-4',9'-dihydro-3'H-spiro[indoline-3,2'-thiopyrano[2,3-b]indole]-2-thione (212)

Yellow powder.

Melting point: 158–160°C.

HPLC t_R = 24.3 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 235, 300, 340.

FTIR (KBr) ν_{max} cm^{-1}: 2926, 1620, 1460,1379.

^{1}H NMR (500 MHz, CDCl$_3$): δ 7.4 (d, J= 10 Hz, 1H), 7.33 (d, J= 10 Hz, 1H), 7.30 (dd, J= 10, 5 Hz, 1H), 7.16 (d, J= 5 Hz, 1H), 7.1 (m, 4H), 7.03 (d, J= 5 Hz, 1H), 6.99 (dd, J= 10, 5 Hz, 1H), 4.12 (s, 3H), 3.88 (s, 3H), 3.33 (dm, 1H), 3.04 (m, 1H), 2.79 (m, 1H), 2.10 (dm, 1H).

^{13}C NMR (500 MHz, CDCl$_3$): δ 193.7, 139.7, 134.2(d), 129.4, 125.6, 125.5, 124.2, 121.8, 120.4(d),117.3, 108.5, 108.2, 103.1, 65.1, 62.2, 60.3, 34.4, 18.2.

HRMS-EI m/z: measured 382.0810 ([M$^+$], calcd. 382.0800 for C$_{20}$H$_{18}$N$_2$O$_2$S$_2$). MS (EI) m/z (% relative int.): 382.08 (28), 351.06 (45), 287.06 (69), 191.03 (49), 161.02 (100).

3.4.2.4 Synthesis of compounds 213 and 214
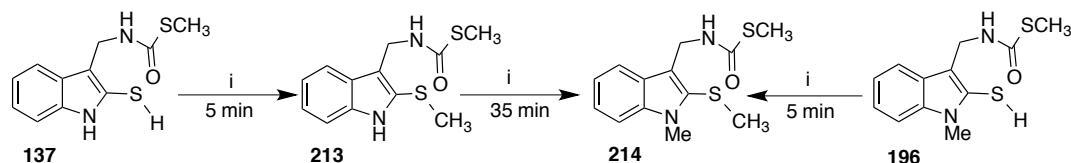
Scheme 3.28 Syntheses of compounds **213** and **214**. Reagents and conditions (i) NaH, MeI, THF, 0 °C, 20% (**213**), 63% (**214**).

Sodium hydride 60% suspension in mineral oil (10 mg, 0.25 mmol, washed with hexane) was added to a cold solution of S-methyl [(2-sulfanyl-1H-indolyl-3)methyl]carbamothioate (**137**, 15 mg, 0.060 mmol) in THF (2 ml) in an ice bath, followed by dropwise addition of MeI (30 μl, 0.10 mmol). After 5 min the reaction mixture was quenched using ice water and extracted with EtOAc. The combined organic extracts were dried and concentrated to dryness. The residue was subjected to FCC (silica gel, CHCl$_3$-hexanes, 7:3) to afford S-methyl [(2-sulfanylmethyl-1H-indolyl-3)methyl]carbamothioate (**213**, 3 mg, 0.01 mmol, 20%). When the same reaction mixture was allowed to proceed for 40 min, compound **214** was obtained in 30% yield (5 mg, 0.02). Compound **214** was prepared similarly from S-methyl [(1-methyl-2-sulfanyl-1H-indolyl-3)methyl]carbamothioate (**196**, 15 mg, 0.060 mmol) to yield S-methyl [(1-methyl-2-sulfanylmethyl-1Hindolyl-3)methyl] carbamothioate (**214**, 10 mg, 0.038 mmol, 63%).

S-methyl [(2-sulfanylmethyl-1H-indolyl-3)methyl]carbamothioate (213)

Brownish gum.

HPLC t_R = 11.3 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 285, 220.

FTIR (KBr) ν_{max} cm^{-1}: 3290, 2927, 2359, 1650, 1505, 1450, 1311, 740.

^{1}H NMR (500 MHz, CDCl$_3$): δ 8.19 (br, 1H), 7.63 (d, J=5 Hz, 1H), 7.34 (d, *J*= 5Hz, 1H), 7.25 (dd, *J*= 10, 5 Hz 1H), 7.16 (dd, *J*= 10, 5, 1H), 5.50 (br, 1H), 4.79 (d, *J*= 5 Hz, 1H), 2.42 (s, 3H), 2.40 (s, 3H).

HRMS-EI *m/z*: measured 266.0539 ([M$^+$], calcd. 266.0548 for C$_{12}$H$_{14}$N$_2$OS$_2$). MS (EI) *m/z* (% relative int.): 266.05 (53), 219.05 (55), 176.05 (100), 117.05 (21).

S-methyl [(1-methyl-2-sulfanylmethyl-1Hindolyl-3)methyl] carbamothioate (214)

Yellowish powder.

Melting point: 146–150 °C.

HPLC t_R = 15.9 min (method A).

UV (HPLC, CH$_3$OH-H$_2$O) λ$_{max}$ (nm): 240, 290.

FTIR (KBr) ν$_{max}$ cm^{-1}: 3310, 2915, 1637, 1516, 1482, 1325, 1209.

^{1}H NMR (500 MHz, CDCl$_3$): δ 7.66 (d, *J*= 5 Hz, 1H), 7.31 (m, 1H), 7.17 (dd, *J*= 10, 5 Hz 1H), 5.47 (br, 1H), 4.84 (s, 1H), 3.87 (s, 3H), 2.39 (s, 3H), 2.30 (s, 3H).

HRMS-EI *m/z*: measured 280.0716 ([M$^+$], calcd. 280.0714 for C$_{13}$H$_{16}$N$_2$OS$_2$). MS (EI) *m/z* (% relative int.): 280.07 (38), 233.08 (70), 190.07 (100).

3.4.3   Rutalexin

3.4.3.1   *Biotransformation of rutalexin*

Time course experiment for rutalexin (**5**) was carried out in MM. Cultures of *A. brassicicola* were prepared as described in **Section 3.4.1.1**. After 48 h of incubation, solution of rutalexin (**5**) (dissolved in DMSO) was added to fungal cultures and to uninoculated MM (triplicate, final concentration in media 0.10 mM). Control cultures of the fungus were grown separately. Samples were withdrawn (5 ml) at different time intervals up to 48 h (0, 6, 12, 24 and 48 h), and extracted with EtOAc (10 ml × 2); the aqueous phase was acidified and reextracted with EtOAc; the remaining aqueous phase was basified and reextracted with CHCl$_3$. All extracts were concentrated and residues were dissolved in CH$_3$CN (0.20 ml) and analyzed by HPLC-DAD-ESI-MS. Similarly, transformation of disulfide **220** was carried out in H$_2$O using a solution of compound in DMSO (final concentration in media 0.10 mM). Samples were withdrawn (5 ml)

at different time intervals and freeze-dried. The residue was dissolved in MeOH-H$_2$O (50:50, 0.20 ml) and analyzed by HPLC-DAD-ESI-MS.

Metabolite **221** resulted from transformation of rutalexin (**5**) by *A. brassicicola* was isolated from the larger scale cultures. Cultures (4 l) were prepared in 250 ml Erlenmeyer flasks containing 100 ml sterile water (culture preparation in water was described in **Section 3.4.1.1**). Rutalexin (**5**, 92 mg, dissolved in DMSO) was added to fungal cultures (final concentration in media 0.10 mM). Cultures were incubated at room temperature for an additional three days. The cultures were filtered and the filtrates were combined (total ca. 4 l) and concentrated using freeze-drier. The concentrated solution (total ca. 100 ml) was extracted with EtOAc (100 ml × 2). The combined extracts were dried and concentrated to dryness to yield an oily residue (160 mg). The residue was subjected to reversed phase column and eluted with CH$_3$CN-H$_2$O (20:80, 15 ml; 25:75, 15 ml; 30:70, 15 ml). All fractions were analyzed by HPLC. The fractions containing the adduct **221** were combined and concentrated to dryness using freeze-dryer to obtain adduct **221** (3.9 mg, 0.010 mmol).
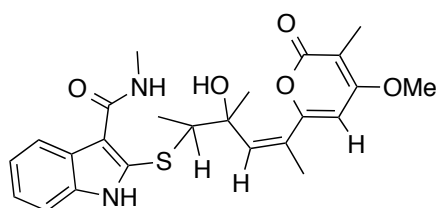
221

HPLC t_R = 9.9 min (method B).
UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 220, 300.
FTIR (KBr) v_{max} cm^{-1}: 1680, 1627, 1551, 1463, 1384, 1226, 1171, 1013, 751.
^{1}H NMR (600 MHz, CH$_3$CN): δ 9.90 (br, 1H), 7.95 (d, *J* =8Hz, 1H), 7.33 (br, 1H), 7.30 (d, *J* =8Hz, 1H), 7.14 (dd, *J* =7, 7Hz, 1H), 7.07 (dd, *J* =7.5, 7.5 Hz 1H), 6.4 (s, 1H), 6.1 (s, 1H), 4.2 (br, 1H), 3.5 (q, *J* = 5Hz, 1H), 2.12 (s, 3H), 2.1 (s, 3H), 1.5 (s, 3H), 1.8 (s, 3H), 1.4 (d, *J* = 7Hz 3H).
^{13}C NMR (600 MHz, CH3CN): δ 167.1, 166.4, 165.2, 160.5, 137.3, 136.8, 130.9, 129.7, 127.8, 124.1, 121.8, 121.7, 115.6, 111.9, 102.5, 94.0, 76.8, 57.6, 57.3, 26.8, 26.4, 17.6, 13.5, 9.0,
HRMS-EI *m/z*: measured 456.1705 ([M]$^+$, calcd. 456.1719 for C$_{24}$H$_{28}$N$_2$O$_5$S) (100%).

3.4.3.2 Synthesis of metabolites
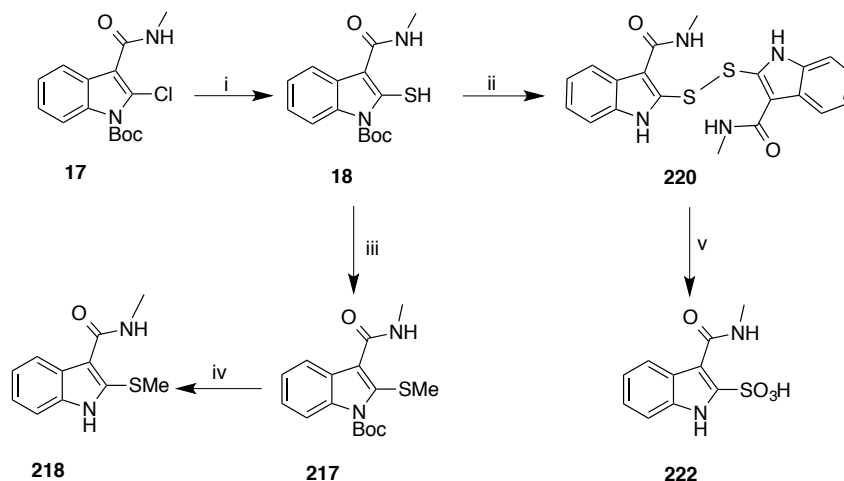
Scheme 3.29 Syntheses of compounds **217**, **218**, **220** and **222**. Reagents and conditions: (i) NaSH, DMF, H$_2$O, 1 h, 0 °C; (ii) 20% TFA in DCM, 4 h, r.t., quantitative; (iii) MeI, THF, 30 min, r.t., 97%; (iv) TFA (20%) in DCM, 4 h, r.t., 94%; (v) *m*-CPBA, DCM, MeOH, 2.5 h, r.t., 59%.

2,2'-disulfanediylbis(N-methyl-1H-indole-3-carboxamide) (220)

A solution of NaSH (108 mg, 1.92 mmol) in water (100 µl) was added to a solution of amide **17** (30 mg, 0.15 mmol) in DMF (1 ml) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and then was diluted with brine (10 ml) and acidified (pH ≤ 3) using HCl (0.50 M). The reaction mixture was extracted with EtOAc. The combined extracts were dried and concentrated to dryness. The residue (**18**) was dissolved in a solution of TFA (20%) in DCM (1.50 ml). The reaction mixture was stirred at room temperature for 4 h and then was concentration to dryness. The residue was rinsed with diethyl ether to give **220** in quantitative yield (20 mg, 0.02 mmol) as white powder.

Melting point: 210 (decomposed)

HPLC t_R = 10.5 min (method B).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 220, 310.

FTIR (KBr) ν_{max} cm^{-1}: 1614, 1535, 1438, 1409, 1209, 1026, 742.

^{1}H NMR (500 MHz, DMSO-d_6): δ 13 (s, 1H), 7.9 (s, br, 1H), 7.8 (d, J= 8Hz, 1H), 7.5 (d, J= 8Hz, 1H), 7.2 (dd, J= 7.5, 7.5Hz, 1H), 7.1 (dd, J= 7.5, 7.5Hz, 1H), 2.9 (d, J= 4.5, 3H).

^{13}C NMR (500 MHz, DMSO-d_6): δ 165.3, 136.8, 134.8, 124.5, 122.7, 120.8, 119.4, 111.7, 111.1, 26.3.

HRMS-EI m/z: measured 410.08847 ([M]$^+$, calcd. 410.08712 for $C_{20}H_{18}N_4O_2S_2$) (100%).

3-(methylcarbamoyl)-1H-indole-2-sulfonic acid (222)

A solution of *m*-CPBA (90 mg, 0.50 mmol) in DCM (1 ml) was added to a solution of disulfide **220** (14 mg, 0.03 mmol) in MeOH (0.50 ml) at room temperature. The reaction mixture was stirred for 2 h at room temperature followed by addition of dimethyl sulfide (0.12 ml, 0.12 mmol). The reaction mixture was kept stirring at room temperature for an additional 30 min followed by concentration to dryness. The residue was fractionated by reversed-phase column (MeOH-H$_2$O) to afford **222** (10 mg, 0.04 mmol, 59%) as white powder.

Melting point: more than 300 °C

HPLC t_R = 1.04 min (method B).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 220, 280.

FTIR (KBr) ν_{max} cm^{-1}: 1600, 1463, 1368, 1118, 1072, 802.

^{1}H NMR (500 MHz, DMSO): δ 11.6 (s, 1H), 9.1 (s, 1H), 8.2 (d, J= 8Hz, 1H), 7.4 (d, J= 8.5Hz, 1H), 7.1 (dd, J =7, 7.5Hz, 1H), 7.0 (dd, J= 7.5, 7.5Hz, 1H), 2.8 (s, 3H).

^{13}C NMR (125.8 MHz, DMSO): δ 164.6, 141.8, 132.9, 127.7, 122.4, 122.4, 120.5, 112.1, 105.8, 25.7.

HRMS-EI m/z: measured 254.0351 ([M]$^+$, calcd. 254.0361 for $C_{10}H_{10}N_2O_4S$) (100%).

N-methyl-2-(methylthio)-1H-indole-3-carboxamide (218)

A solution of NaSH (108 mg, 1.19 mmol) in water (100 μl) was added to a solution of amide **17** (30 mg, 0.15 mmol) in DMF (1 ml) at 0 °C. The reaction mixture was stirring at 0 °C

for 1h, followed by dropwise addition of methyl iodide (187 μl, 3 mmol). The ice bath was removed and the reaction mixture was stirred at room temperature for an additional 30 min followed by dilution with water and extraction with EtOAc. The combined extracts were dried and concentrated to dryness to afford *tert*-butyl 3-(methylcarbamoyl)-2-(methylthio)-1*H*-indole-1-carboxylate (**217**, 24 mg, in 97%) as a light yellowish powder.

HPLC t_R = 13.4 min (method B).

UV (HPLC, CH_3OH-H_2O) λ_{max} (nm): 212, 293.

FTIR (KBr) ν_{max} cm^{-1}: 1736, 1639, 1446, 1370, 1321, 1154, 1101, 748.

^{1}H NMR (500 MHz, CDCl$_3$): δ 8.3 (d, *J*= 7.5 Hz, 1H), 7.9 (d, *J*= 8.5 Hz, 1H), 7.7 (br. 1H), 7.3 (dd, *J*= 10, 5 Hz, 1H), 7.3 (dd, *J*= 10, 5 Hz, 1H), 3.1 (d, *J*= 4.5 Hz, 3H), 2.5 (s, 3H), 1.7 (s, 9H).

^{13}C NMR (500 MHz, CDCl$_3$): δ 165.0, 149.6, 136.5, 132.2, 128.0, 125.8, 123.9, 122.4, 121.2, 114.4, 85.7, 28.4, 26.5, 21.2.

HRMS-EI *m/z*: measured 320.11966 ([M]$^+$, calcd. 320.11946 for C$_{16}$H$_{20}$N$_2$O$_3$S) (100%).

A mixture of TFA (20%) in DCM (2 ml) was added to a solution of *tert*-butyl 3-(methylcarbamoyl)-2-(methylthio)-1*H*-indole-1-carboxylate (**217**, 24 mg, 0.080 mmol) in DCM. The reaction mixture was stirred at room temperature for 4 h and then was concentrated to dryness. The residue was subjected to FCC (silica gel, EtOAc-hexane, 1:1) to afford *N*-methyl-2-(methylthio)-1*H*-indole-3-carboxamide (**218**) as white powder in 94% yield (15mg, 0.070 mmol).

HPLC t_R = 2.8 min (method B).

UV (HPLC, CH_3OH-H_2O) λ_{max} (nm): 220, 300.

FTIR (KBr) ν_{max} cm^{-1}: 2926, 1725, 1618, 1543, 1413, 1313, 1148, 750.

^{1}H NMR (500 MHz, CDCl$_3$): δ 8.8 (br, 1H), 8.3 (d, *J*= 7 Hz, 1H), 7.3 (d, *J*= 7 Hz, 1H), 7.2 (m, 2H), 3 (d, *J*= 3.5Hz, 3H), 2.5 (s, 3H).

^{13}C NMR (500 MHz, CDCl$_3$): δ 166.3, 136.3, 132.2, 127.6, 123.5, 121.8, 121.6, 112.3, 111.0, 26.5, 19.3.

HRMS-EI *m/z*: measured 220.06678 ([M]$^+$, calcd. 220.06703 for C$_{11}$H$_{12}$N$_2$OS) (100%).

3.4.4 Brassilexin and 1-methylbrassilexin

3.4.4.1 *Biotransformation of brassilexin and 1-methylbrassilexin*

Time course experiments for brassilexins **7**, **165** were carried out in MM following the procedure described in **Section 3.4.1.1**, using a final concentration of 0.10 mM of each compound in the cultures and controls. Cultures were incubated; samples were withdrawn (5 ml) up to 48 h (0, 6, 12, 24 and 48 h), and treated as reported in **Section 3.4.1.1**.

3.4.4.2 *Synthesis of metabolites*

Synthesis of enamines 99 and 228

Synthesis of enamines **99** and **228** as an intermediate in synthesis of brassilexin (**7**) and 1-methylbrassilexin (**165**) respectively, were described in **Section 3.3.1.5**.

Synthesis of compounds 226 and 227

224 R=H
225 R=Me

226 R=H
227 R=Me
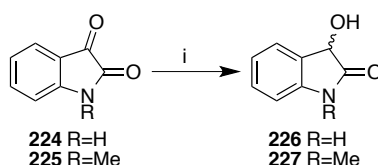
Scheme 3.30 Syntheses of compounds **226** and **227**. Reagent and condition: (i) NaBH$_4$, 95% EtOH, 20 min, r.t., 68% (**226**) and 92% (**227**), (Bergonzini and Melchiorre, 2011).

Isatin (**224**, 147 mg, 1.00 mmol) was added to a solution of NaBH$_4$ (111 mg, 3.00 mmol) in EtOH (95%, 10 ml). The reaction mixture was stirred at room temperature for 20 min. The resulting suspension was purred into cold water (30 ml) in an ice bath, acidified (HCl, pH ~ 5) and extracted with chloroform. The combined extracts were dried and concentrated to dryness. The residue was subjected to FCC (silica gel, DCM-MeOH, 98:2.0) to afford **226** (100 mg, 68%) as yellow powder. Similarly compound **227** was synthesized using 1-Methylisatin (161 mg, 1.00

mmol) and NaBH$_4$ (111 mg, 3.00 mmol) to afford **227** (150 mg, 92%) as a yellow powder (Bergonzini and Melchiorre, 2011).

3-hydroxyindoline-2-one (226)

Melting point: 118–120 °C

HPLC t_R = 2.7 min (method C).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 210, 260, 300.

FTIR (KBr) ν_{max} cm^{-1}: 1717, 1623, 1469, 1266, 1215, 743.

^{1}H NMR (500 MHz, MeOD): δ 7.4 (d, J= 7 Hz, 1H), 7.24 (dd, J= 8, 7.5 Hz, 1H), 7 (dd, J= 8, 7.5 Hz, 1H), 6.9 (d, J= 8 Hz, 1H), 4.9 (s, 1H).

^{13}C NMR (500 MHz, MeOD): δ 180.8, 143.4, 130.7, 130.3, 126.2, 123.7, 111.3, 71.3.

HRMS-EI m/z: measured 149.0474 ([M]$^+$, calcd. 149.0474 for C$_8$H$_7$N$_1$O$_2$) (90.9%).

3-hydroxy-1-methylindoline-2-one (227)

Melting point: 148–150 °C

HPLC t_R = 4.6 min (method C).

UV (HPLC, CH$_3$OH-H$_2$O) λ_{max} (nm): 210, 260, 300.

FTIR (KBr) ν_{max} cm^{-1}: 3293, 1702, 1619, 1469, 1385, 1269, 1090, 758.

^{1}H NMR (500 MHz, CD$_3$Cl): δ 7.4 (d, J= 7 Hz, 1H), 7.3 (dd, J= 7.5, 8 Hz, 1H), 7.1 (dd, J= 7.5, 7.5 Hz, 1H), 6.9 (d, J= 7.5 Hz, 1H), 4.9 (s, 1H), 3.1 (s, 3H).

^{13}C NMR (500 MHz, MeOD): δ 178.6, 145.2, 130.8, 129.6, 125.8, 124.2, 109.9, 70.9, 26.5.

HRMS-EI m/z: measured 163.0630 ([M]$^+$, calcd. 163.0633 for C$_9$H$_9$N$_1$O$_2$) (100%).

3.5   Screening of potential brassinin detoxification inhibitors using cell free extracts

3.5.1   Fungal culture of Leptosphaeria maculans

Liquid cultures of *L. maculans* were initiated by inoculating MM (100 ml) with fungal spores at 10^7/ml in 250 ml Erlenmeyer flasks, followed by incubation in a shaker under constant light at 23 °C. Two days old cultures of *L. maculans* were incubated with 3-phenylindole (0.10 mM final concentration in cultures to induce BO) for an additional 24 h and then gravity filtered to separate mycelia from the culture broth. The mycelia was dried by squeezing and was stored at -20 °C and used to obtain protein extracts containing BO activity.

3.5.2   Preparation of crude cell free extract

Frozen mycelia (1.2 g) from *L. maculans* were suspended in ice-cold extraction buffer (5 ml) and ground (mortar) for 5 min at 4 °C. The extraction buffer consisted of diethanolamine (DEA, 25 mM, pH ~ 8.3), 10% (v/v) glycerol, D,L-dithiothreitol (DTT, 1 mM), and 1/200 (v/ v) protease inhibitor cocktail (P-8215, Sigma-Aldrich Canada). The homogenate was centrifuged at 4 °C for 30 min at 50000 g. The resulting supernatant was dialyzed 3 times (2 times with 300 ml of dialyzing buffer for 3h in each time and then using 400 ml buffer for 12 h) using dialyzing cassettes in buffer (diethanolamine (DEA, 25 mM, pH ~ 8.3), 5% (v/v) glycerol, triton X-100 (10%) and deionized water). Dialyzed cell-free extract was used for determination of specific activity of BO. Protein concentrations were determined as described by Bradford using the Coomassie Brilliant Blue method with BSA as a standard.

3.5.3   Protein measurement

In a spectrophotometric cell (1 ml) were taken 5.00 µl of Protein extract and 995 µl of Bradford reagent. After mixing the solution, mixture was incubated for 5 min in dark condition and the optical density (OD) was measured at 595 nm. A blank sample containing 5.00 µl

extraction buffer and 995 μl Bradford reagent was used as control. All samples were prepared in triplicate and finally the concentration of proteins was determined using the BSA calibration curve.

3.5.4 Preparation of BSA calibration curve

Calibration curve was prepared from bovine serum albumin (BSA). The calibration curve was used to estimate the amount of protein in the cell homogenate. Different concentrations of BSA (0.300, 0.250, 0.200, 0.150 and 0.100 mg/ml) were prepared using the serial dilution from the stock solution of BSA in extraction buffer (1mg/ml). 100 μl of each solution together with 900 μl of Bradford reagent were added to a spectrophotometric cell (1 ml). Resulted solution was mixed well and incubated for 5 min. The OD was measured at 595 nm. A blank sample containing 100 μl of extraction buffer and 900 μl of Bradford reagent was used as control. All samples were prepared in triplicate. The calibration curve was obtained by plotting concentration *vs* OD.

3.5.5 Enzyme assay

The reaction mixture contained DEA (20 mM, pH ~ 8.3), DTT (0.10 mM), 0.1% (v/v) triton X-100, brassinin (0.10 mM), phenazine (0.10 mM), and protein extract (50 μl) in a total volume of 1.0 ml. The reaction was carried out at 24 °C for 20 min. The product was extracted with EtOAc (4 ml) and concentrated to dryness. The extract was dissolved in CH_3CN (200 μl) and analyzed by HPLC-DAD. The amount of indole-3-carboxaldehyde (**26**) in the reaction assay was determined using calibration curve built with pure indole-3-carboxaldehyde (**26**). One enzyme unit (U) is defined as the amount of the enzyme that catalyzes the conversion of one micromole of substrate per minute ($\mu mol \cdot min^{-1} = U$).

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