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

A Thesis Submitted to the
College of Graduate Studies and Research
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the
Department of Chemistry
University of Saskatchewan
Saskatoon

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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

I would like to express my sincere gratitude to my supervisor Prof. M. Soledade C. Pedras for her mentorship, patience, support and guidance, without which this thesis would not have been possible. Her vast knowledge in the multiple disciplines and her professionalism has been of great value for me. Her dedication to work and passion in science will remain as an inspiration for me.

I am also grateful to the members of my Advisory Committee: Prof. D. E. Ward and Dr. S. Reid Department of Chemistry, University of Saskatchewan; and Dr. J. Balsevich National Research Council, Saskatoon Saskatchewan. Their valuable advice and help during my PhD. work is greatly acknowledged. I also thank my external examiner, Prof. Andrew G. H Wee, Department of Chemistry and Biochemistry University of Regina, for his review of my thesis, suggestions and advice.

I would like to acknowledge the support and encouragement from all past and present members of Prof. Pedras group: Dr. P. B. Chumala, Dr. Z. Minic, Dr. S. Hossain, Dr. V. K. Sarma-Mamillapalle, Dr. I. Khallaf, Dr. E. YAYA, Dr. M. Y. Park, Dr. M. N. Khan, Dr. S. M. A. Hussaini, M. Alavi, C. Thapa and H. To and I also wish to extend my warmest thanks to K. Thoms, Dr. K. Brown, and Dr. G. Schatte for their technical assistance.

I express my heart felt gratitude to my wife Fatemeh Alhosseini and my father, mother, brothers, sisters and their family members for their loving support and encouragement.

Finally I wish to acknowledge the Department of Chemistry and the College of Graduate Studies and Research, University of Saskatchewan for financial support.
DEDICATION

to

My Mother, Father

and

My wife
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<th>Abbreviation / Symbol</th>
<th>Full Form</th>
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<td>acetyl</td>
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<td><em>Alternaria brassicicola</em></td>
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<td><em>Aspergillus oryzae</em></td>
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<tr>
<td>A. thaliana</td>
<td><em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>Ac$_2$O</td>
<td>acetic anhydride</td>
</tr>
<tr>
<td>AcOH</td>
<td>acetic acid</td>
</tr>
<tr>
<td>AIBN</td>
<td>azobisisobutyronitrile</td>
</tr>
<tr>
<td>br</td>
<td>broad</td>
</tr>
<tr>
<td>B.</td>
<td><em>Brassica</em></td>
</tr>
<tr>
<td>B. bassiana</td>
<td><em>Beauveria bassiana</em></td>
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<td>B. cinerea</td>
<td><em>Botrytis cinerea</em></td>
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<td>BHAb</td>
<td>brassinin hydrolase from <em>A. brassicicola</em></td>
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<td>BHLmL2</td>
<td>brassinin hydrolase from <em>L. maculans</em> (Laird-2)</td>
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<td>brassinin oxidase</td>
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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:

- Investigate the metabolism and determine the products of transformation of the cruciferous phytoalexins camalexin (1), 1-methylcamalexin (2), 6-methoxycamalexin (3), cyclobassin (4), rutalexin (5), brassilexin (7), and rapalexin A (8) by Alternaria brassicicola;
- Investigate the metabolism and determine the products of transformation of the camalexin related structures, 1-methylcyclobassin (193) and 1-methylbrassilexin (165) by Alternaria brassicicola;
- Determine the antifungal activity of phytoalexins, selected compounds and metabolites resulting from biotransformation against Alternaria brassicicola;
- 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 brassicaceae* (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 brassicaceae* (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.
Cyclobrassinin (4) was first isolated from Chinese cabbage (*Brassica campestris* L. ssp. *pekinesis*) 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. brassicaceae, 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) NaClO₂, 2-methylbut-2-ene, KH₂PO₄, tert-butyl alcohol/H₂O, r.t., 2 h, 96%; (ii) SOCl₂, THF, rt, 3 h, then CH₃NH₂, THF, 0 °C, 20 min, 85% (based on acid 16); (iii) NaSH, DMF/H₂O, 0 °C, 2 h; (iv) NaH, THF, 0 °C, 10 min then COCl₂ (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/H₂O (1:1, cat.), acetone, r.t., overnight, 80%; (ii) DBU, CH₃I, THF, under N₂, r.t., 2 h, 95%; (iii) 165–170 °C, 30 min, quant.; (iv) Boc-anhydride, DMAP, THF, 5 °C, 1 h, 88%; (v) PCC, CH₂Cl₂, 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) Boc₂O, 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) CH₃OH, acetone, r.t., 2 h; (v) Et₃N, 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 AgNO₃ 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) AgNO₃, benzoyl chloride, CH₃CN, 30%; (ii) Pd/C, H₂, AcOH; (iii) CH₂Cl₂, CaCO₃, CSCl₂, 20% over two steps; (Pedras et al., 2007b); (iv) TFA, TTFA, I₂, 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).
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$_3$N, 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$_3$N, 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$_2$Cl$_2$, 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$_3$N, 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).
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).
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
isolated from liquid cultures of *A. brassicicola* and established to be a host-selective toxin (HST) (Pedras et al., 2009a).

![Chemical structures of brassicicolin A (56) and depudecin (57).](image)

**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).
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*.

**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).
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) LiAlH₄, Et₂O, 0 °C, 77%; (ii) PBr₃, Et₂O, 0 °C, 51%; (iii) Ph₃P, CH₃CN, 120 °C, 75%; (iv) BuLi, THF, 0 °C, 78% (Geiseler and Podlech, 2012).

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

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 ($\Delta$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.
Nα-dimethyl coprogen

**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* (*AbSlit2Δ*) deficient in production of a kinase (*Slit2*) 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) (*Δbdtfl*) 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 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](image)

**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).
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 \( N_\beta \)-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).
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 1.20** 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).

**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](image)

**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 chrysogenum* was investigated (Herath et al., 2013). Resveratrol (119) was metabolized by *B.*
Beauveria bassiana to 5-methoxyresveratrol-3-O-glucoside (120) and 5-hydroxyresveratrol-3-sulphate (122) (Herath et al., 2013). P. chrysogenum 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 chrysogenum (Herath et al., 2013).](image)

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).
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).

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).
1.4.1 Detoxifying 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).

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

![Scheme 1.28 Transformation of brassinin (9) by brassinin oxidase from Leptosphaeria maculans (isolate virulent on canola) (BOLm); Brassinin hydrolase from Alternaria brassicicola (BHA); brassinin hydrolase from Leptosphaeria maculans (virulent on mustard) (BHLmL2) and brassinin glucosyl transferase from Sclerotinia sclerotiorum (SsBG1).]
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](image-url) Structures of phytoalexin that are inhibitors of phytoalexin detoxifying enzymes.
<table>
<thead>
<tr>
<th>Compound (#)</th>
<th>Inhibition (%)</th>
<th>Conc. (mM)</th>
<th>BOLm</th>
<th>BHLmL2</th>
<th>BHAb</th>
<th>CHAb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camalexin (1)</td>
<td></td>
<td>0.30</td>
<td>53 ± 4</td>
<td>n. d.</td>
<td>n. d.</td>
<td>65 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>30 ± 4</td>
<td>n. d.</td>
<td>n. d.</td>
<td>52 ± 6</td>
</tr>
<tr>
<td>6-Methoxycamalexin (3)</td>
<td></td>
<td>0.30</td>
<td>63 ± 5</td>
<td>n. d.</td>
<td>n. d.</td>
<td>n. d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>41 ± 6</td>
<td>n. d.</td>
<td>n. d.</td>
<td>n. d.</td>
</tr>
<tr>
<td>1-Methylcamalexin (2)</td>
<td></td>
<td>0.30</td>
<td>n. i.</td>
<td>n. d.</td>
<td>n. d.</td>
<td>n. d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>n. i.</td>
<td>n. d.</td>
<td>n. d.</td>
<td>n. d.</td>
</tr>
<tr>
<td>Cyclobrassinin (4)</td>
<td></td>
<td>0.30</td>
<td>37 ± 8</td>
<td>n. d.</td>
<td>26 ± 4</td>
<td>n. d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>23 ± 6</td>
<td>n. i.</td>
<td>16 ± 2</td>
<td>n. d.</td>
</tr>
<tr>
<td>Brassilexin (7)</td>
<td></td>
<td>0.30</td>
<td>16 ± 2</td>
<td>n. i.</td>
<td>n. i.</td>
<td>93 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>8 ± 2</td>
<td>n. i.</td>
<td>n. i.</td>
<td>85 ± 2</td>
</tr>
<tr>
<td>Wasalexin A &amp; B (138 &amp; 139)</td>
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<td>0.30</td>
<td>14 ± 4</td>
<td>n. d.</td>
<td>n. d.</td>
<td>n. d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>8 ± 5</td>
<td>n. i.</td>
<td>n. i.</td>
<td>n. i.</td>
</tr>
<tr>
<td>Rutalexin (5)</td>
<td></td>
<td>0.30</td>
<td>n. d.</td>
<td>n. i.</td>
<td>n. i.</td>
<td>n. d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>n. i.</td>
<td>n. i.</td>
<td>n. i.</td>
<td>n. i.</td>
</tr>
<tr>
<td>Erucalexin (13)</td>
<td></td>
<td>0.30</td>
<td>n. d.</td>
<td>n. i.</td>
<td>n. i.</td>
<td>n. d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>n. i.</td>
<td>n. i.</td>
<td>n. i.</td>
<td>n. i.</td>
</tr>
<tr>
<td>Brassicanal A (6)</td>
<td></td>
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<td>n. d.</td>
<td>n. i.</td>
<td>n. i.</td>
<td>43 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>n. i.</td>
<td>n. i.</td>
<td>n. i.</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Brassinin (9)</td>
<td></td>
<td>0.30</td>
<td>n. d.</td>
<td>n. d.</td>
<td>n. d.</td>
<td>n. i.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>n. i.</td>
<td>n. i.</td>
<td>n. i.</td>
<td>n. i.</td>
</tr>
<tr>
<td>Sinalexin (140)</td>
<td></td>
<td>0.30</td>
<td>n. d.</td>
<td>n. d.</td>
<td>n. d.</td>
<td>52 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>n. i.</td>
<td>n. i.</td>
<td>n. i.</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>Spirobrassinin (11)</td>
<td></td>
<td>0.30</td>
<td>n. d.</td>
<td>n. d.</td>
<td>n. d.</td>
<td>n. i.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>n. i.</td>
<td>n. i.</td>
<td>n. i.</td>
<td>n. i.</td>
</tr>
<tr>
<td>Dioxibrasinin (103)</td>
<td></td>
<td>0.30</td>
<td>n. d.</td>
<td>n. d.</td>
<td>n. d.</td>
<td>58 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>n. i.</td>
<td>n. i.</td>
<td>n. i.</td>
<td>40 ± 5</td>
</tr>
</tbody>
</table>

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 extent 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-substituted brassilexins (Pedras et al., 2010a).

![Figure 1.12](image-url) 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](image-url) 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).

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).
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-(1H-indol-3-yl)-4-methylthiazole (173) and 2-(1H-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) Br₂, 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 H₂/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) NaN₃, H₂O, acetone, 50 °C, 20 h, 81%; (ii) Pd/C, H₂, HCl, MeOH, r.t., 3 h, 85%; (iii) Ethyl formate, Et₃N, 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 POCl₃/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) POCl₃, DMF, 0-90 °C, 3 h, 91%; (ii) NaOH, 1,4-dioxane, reflux, 8 h, 90%; (iii) SOCl₂, THF, -20 °C, 10 min; (iv) NH₄SCN, 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*.

<table>
<thead>
<tr>
<th>Compound (#)</th>
<th>Inhibition ± SD (%)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>0.50mM</td>
</tr>
<tr>
<td>Camalexin (1)</td>
<td>-</td>
</tr>
<tr>
<td>1-Methycamalexin (2)</td>
<td>-</td>
</tr>
<tr>
<td>6-Methoxycamalexin (3)</td>
<td>100 ± 0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4′-Methylcamalexin (173)</td>
<td>-</td>
</tr>
<tr>
<td>5′-Methylcamalexin (174)</td>
<td>-</td>
</tr>
<tr>
<td>1H-3-(4′-Thiazolyl)indole (169)</td>
<td>100 ± 0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1H-3-(5′-Thiazolyl)indole (170)</td>
<td>-</td>
</tr>
<tr>
<td>1H-3-(4′-Isothiazolyl)indole (168)</td>
<td>100 ± 0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1H-3-(2′-Oxazolyl)indole (167)</td>
<td>100 ± 0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1H-3-(2′-Thia-3′,4′-diazolyl)indole (172)</td>
<td>100 ± 0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1H-3-(2′-Oxa-3′,4′-diazolyl)indole (171)</td>
<td>53 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
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</table>

<sup>a</sup>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 1H-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).
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*<sub>R</sub> = 4.6 min), indole-3-carbonitrile (83, *t*<sub>R</sub> = 7.8 min) and indole-3-carboxylic acid (84, *t*<sub>R</sub> = 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).

![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.](image-url)
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.
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-(1H-indol-3-yl)thiazol-4-yl)methnol (185, ■) in *Alternaria brassicicola* and in control medium (□).

![Curves](image)

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

![Structures](image)

Time-course studies revealed that 5ʹ-methylcamalexin (174) was almost completely metabolized within 96 h by *A. brassicicola* at a rate comparable to 4ʹ-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 5ʹ-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-(1H-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-(1H-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 1H-3-(2′-oxazolyl)indole (167) and 2-(1H-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.
Figure 2.14  Structure of indole-3-carboxamide (186) from transformation of 2-(1H-indol-3-yl)-1,3,4-oxadiazole (171) by Alternaria brassicicola.

Figure 2.15  Progress curves of transformation of 1H-3-(2′-oxazolyl)indole (167, ●) by Alternaria brassicicola and formation of 1H-indole-3-carboxamide (186, □) and recovery of 1H-3-(2′-oxazolyl)indole (167, ○) in control medium.

Figure 2.16  Progress curves of transformation of 2-(1H-indol-3-yl)-1,3,4-oxadiazole (171, ■) by Alternaria brassicicola and formation of 1H-indole-3-carboxamide (186, ▲) and recovery of 2-(1H-indol-3-yl)-1,3,4-oxadiazole (171, □) in control medium.
The HPLC-DAD-ESI-MS chromatograms of neutral extracts of cultures of *A. brassicicola* incubated with 1H-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. 1H-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](image)

**Figure 2.17** Progress curves of transformation of 1H-3-(2’-thia-3’,4’-diazolyl)indole (172, ■) by *Alternaria brassicicola* and formation of indole-3-thiocarboxamide (82, ●) and indole-3-carboxylic acid (84, ▲) and recovery of 1H-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 1H-3-(4’-thiazolyl)indole (169), 1H-3-(5’-thiazolyl)indole (170) and 1H-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 (◇).
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).

**Figure 2.20** Curves representing the recovery of 170 in cultures of *Alternaria brassicicola* in minimal medium (■) and in control medium (□).
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) POCl3, DMF, r.t., 2.5 h, 98%; (ii) HONH2.HCl, NaOAc, 95% EtOH, H2O, r.t., 3 h; (iii) Ac2O, pyridine, CH2Cl2, 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 (NaClO2) under mild acidic condition (Scheme 2.5) as described in Section 3.4.1.3.4 (Pedras and Abdoli, 2013).

Scheme 2.5 Syntheses of acids 94 and 95. Reagents and conditions: (i) NaClO2, NaH2PO4, t-butanol, 2-methylbut-2-ene, H2O, 14 h, r.t., 75% (94) and 60 h, 52% (95).
The new compounds 1H-3-(4-hydroxymethyl-2-thiazolyl)indole (185) and 2-(1H-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 LiAlH₄ 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) LiAlH₄, 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*.

<table>
<thead>
<tr>
<th>Compound (#)</th>
<th>Inhibition ± SD (%)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>0.50 mM</td>
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<tr>
<td>Indole-3-thiocarboxamide (82)</td>
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<tr>
<td>Indole-3-carbonitride (83)</td>
<td>100 ± 0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Indole-3-carboxylic acid (84)</td>
<td>26 ± 2&lt;sup&gt;h&lt;/sup&gt;</td>
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<tr>
<td>1-Methylindole-3-thiocarboxamide (90)</td>
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<td>2-1H-3-(4-Hydroxymethyl-2-thiazolyl)indole (185)</td>
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<tr>
<td>Indole-3-carboxamide (186)</td>
<td>68 ± 1&lt;sup&gt;d&lt;/sup&gt;</td>
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</table>

<sup>a</sup>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 1H-3-(4-hydroxymethyl-2-thiazolyl)indole (173) and 2-(1H-indol-3-yl)-4-thiazolecarboxylic acid (174) by *Alternaria brassicicola* and metabolic products.

**Figure 2.21** Progress curves of transformations of 1H-3-(4-hydroxymethyl-2-thiazolyl)indole (173) (○) and 2-(1H-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 1H-3-(2’-oxazolyl)indole (167) and 1H-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 1H-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 $1H$-$3$-(2′-oxazolyl)indole (167, ▲), $1H$-$3$–(2′-oxa-3′,4′-diazolyl)indole (171, ●) and $1H$-$3$-(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).

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*.

<table>
<thead>
<tr>
<th>Compound (#)</th>
<th>Inhibition ± SD (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.50 mM</td>
</tr>
<tr>
<td>Cyclobrassinin (4)</td>
<td>39 ± 3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1-Methylcyclobrassinin (193)</td>
<td>69 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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 (<sup>b</sup>–<sup>e</sup>) 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.

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

**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-1H-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-H₂O (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.

![Figure 2.27](image_url)  
**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 $\delta$ 3.7 ppm. Considering the $^1$H NMR data, structure 196 was proposed for the metabolite at 18.8 min (Figure 2.29).

![Structure 196](image)

Figure 2.29 Structures of S-methyl [(1-methyl-2-sulfanyl-1H-indolyl-3)methyl]carbamothioate (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.
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 ($\delta$H 7.0-8.5), two were for a methylene group ($\delta$H 3.9, s, 2H) and three were for methyl groups ($\delta$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.

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-$d_6$).

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

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_{2}S_{2}, as suggested by HRMS-EI. The $^{1}$H NMR (CDCl$_{3}$) spectrum of the metabolite at 17.9 min displayed signals for 14 protons: eight aromatic ($\delta_{H}$ 7.0-7.5), two D$_{2}$O exchangeable ($\delta_{H}$ 9.51 and 7.94) and four methylenic protons ($\delta_{H}$ 3.65, d, $J$=16 Hz, 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 $\delta_{H}$ 3.65 correlated to the C=S at $\delta_{C}$ 210.8 and that methylene protons at $\delta_{H}$ 3.65 and 2.83 correlated with a quaternary carbon at $\delta_{C}$ 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.

![Figure 2.32](image_url) 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$_{2}$S$_{2}$) obtained from HRMS (EI and ESI) and NMR spectral data. Similar to compound 201, obtained from hydrolysis of cyclobrassinin (4), the $^{1}$H 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 ($\delta_{H}$ 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 $\delta_C 208.6$ was assigned to a thiocarbonyl group. This spectroscopic analysis suggested structure 203 for one of the isomers (Pedras et al., 2013). The $^1H$ 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 ($\delta_H 3.33$, ddd, $J = 17$, 12, 6 Hz and 3.10, ddd, $J = 17$, 12, 6 Hz; 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 $\delta_H 3.33$ correlated with carbon at $\delta_C 104.9$ and a quaternary sp$^3$ carbon at $\delta_C 62.5$, as summarized in Figure 2.33.

![202](image)

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

![Scheme 2.12](image)

**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.

![Figure 2.34](image.png)

**Figure 2.34** Structures of 3-methyleneindoline-2-thiones 205 and 206.

![Scheme 2.13](image.png)

**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).

![Scheme 2.14](image.png)

**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**).
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>
<thead>
<tr>
<th>Indoline-2-thiones</th>
<th>Conditions</th>
<th>Products (ratio)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>207</td>
<td>HCl</td>
<td>203+202 (1:1)</td>
<td>32</td>
</tr>
<tr>
<td>208</td>
<td>HCl</td>
<td>211+212 (1.2:1)</td>
<td>46</td>
</tr>
<tr>
<td>207</td>
<td>TFA</td>
<td>203+202 (1:1)</td>
<td>20</td>
</tr>
<tr>
<td>208</td>
<td>TFA</td>
<td>211+212 (1.2:1)</td>
<td>33</td>
</tr>
</tbody>
</table>

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-Et₂O 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$_3$).

<table>
<thead>
<tr>
<th>C/H #</th>
<th>$\delta$C</th>
<th>$\delta$H (m, J, H)</th>
<th>$\delta$C</th>
<th>$\delta$H (m, J, H)</th>
<th>$\delta$C</th>
<th>$\delta$H (m, J, H)</th>
<th>$\delta$C</th>
<th>$\delta$H (m, J, H)</th>
<th>$\delta$C</th>
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<td>-</td>
<td>208.6</td>
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<td>210.8</td>
<td>-</td>
<td>198.4</td>
<td>-</td>
<td>193.7</td>
<td>-</td>
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<tr>
<td>3</td>
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</tr>
<tr>
<td>3a</td>
<td>136.5</td>
<td>-</td>
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<td>-</td>
<td>135.7</td>
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<td>124.2</td>
<td>7.11 (d, 7.5 Hz, 1H)</td>
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<td>7.23 (d, 7.5 Hz, 1H)</td>
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<td>7.03 (dd, 7.5, 7.5 Hz, 1H)</td>
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<td>140.3</td>
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<td>139.7</td>
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</tr>
<tr>
<td>3'</td>
<td>18.5</td>
<td>3.33 (ddd, 17, 12, 6 Hz, 1H)</td>
<td>32.9</td>
<td>3.64 (d, 16 Hz, 1H)</td>
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<td>3.65 (d, 16 Hz, 1H)</td>
<td>31.9</td>
<td>3.86 (d, 15 Hz, 1H)</td>
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<td></td>
<td>2.77 (dd, 16, 2 Hz, 1H)</td>
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<td>2.83 (dd, 16, 2 Hz, 1H)</td>
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<td>2.73 (d, 15 Hz, 1H)</td>
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<td>3.78 (s, 3H)</td>
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<td>7.94 (s, N-H)</td>
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<td>127.6</td>
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<td>-</td>
<td>124.2</td>
<td>-</td>
</tr>
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<td>103.1</td>
<td>-</td>
</tr>
<tr>
<td>4'b</td>
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<td>-</td>
<td>128.1</td>
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</tr>
<tr>
<td>C/H #</td>
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<td>δ_H (m, J, H) 202</td>
<td>δ_C 203</td>
<td>δ_H (m, J, H) 203</td>
<td>δ_C 201</td>
<td>δ_H (m, J, H) 201</td>
<td>δ_C 211</td>
<td>δ_H (m, J, H) 211</td>
<td>δ_C 212</td>
<td>δ_H (m, J, H) 212</td>
</tr>
<tr>
<td>-------</td>
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</tr>
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<td>125.5</td>
<td>6.99 (dd, 10, 5 Hz, 1H)</td>
</tr>
<tr>
<td>7'</td>
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</tr>
<tr>
<td>8'</td>
<td>108.4</td>
<td>7.30 (d, 7.5 Hz, 1H)</td>
<td>108.2</td>
<td>7.31 (d, 7.5 Hz, 1H)</td>
<td>110.1</td>
<td>7.36 (d, 7.5 Hz, 1H)</td>
<td>107.2</td>
<td>7.22 (m, 3H)</td>
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<td>-</td>
<td>137.5</td>
<td>136.5</td>
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<td>132.4</td>
<td>-</td>
<td>134.2</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>32.1</td>
<td>3.74 (s, 3H)</td>
<td>30.1</td>
<td>3.77 (s, 3H)</td>
<td>9.51</td>
<td>9.51 (s, N-H)</td>
<td>62.2</td>
<td>4.1 (s, 3H)</td>
<td>62.2</td>
<td>3.88 (s, 3H)</td>
</tr>
</tbody>
</table>
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 H2O2. Compound 194 was obtained by oxidation of compound 137 with H2O2 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., H2O2 (16.5eq), 16 h, 194 (91%) or H2O2 (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. 1H NMR (DMSO-d6) spectrum of the residue displayed signals for 11 protons, of which six were aromatic (δH 7.1-8.2), two were from a methylene group (δH 3.9, s, 2H) and three were from a methyl group (δH 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 H₂O₂ gave sulfonic acid 195 as the major compound. ¹H 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.

<table>
<thead>
<tr>
<th>Compound (#)</th>
<th>Inhibition ± SD (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.50 mM</td>
</tr>
<tr>
<td>Hyd-cyclobrassinin (137)</td>
<td>14 ± 3&lt;sup&gt;c,f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hyd-methylcyclobrassinin (196)</td>
<td>24 ± 0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Methylspiro (203)</td>
<td>27 ± 3&lt;sup&gt;b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Methylspiro (202)</td>
<td>25 ± 0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Methoxyspiro (211)</td>
<td>33 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Methoxyspiro (212)</td>
<td>25 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>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 (<sup>b–g</sup>) 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 (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-methycyclobrassinin (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-methycyclobrassinin (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-methycyclobrassinin (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](image)

**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-methylocyclobrassinin (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 $2 \times 10^{-6}$ mol (Pedras et al., 2004b).

![Structure of rutalexin (5).](image)

**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 Et₃N followed by deprotection using TFA yielded crude rutalexin (5). The final reaction mixture was concentrated to dryness and the residue was rinsed with Et₂O 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$_2$PO$_4$, 2-methylbut-2-ene, $t$-butanol, H$_2$O, r.t., 100%; (ii) SOCl$_2$, DMF, THF, r.t.; iii) CH$_3$NH$_2$, THF, 0 °C, 95% (over steps ii and iii); (iv) NaSH, DMF, H$_2$O, 0 °C, 1 h; (v) Methyl chloroformate, 30 min, 216 (95%) and 6 h, 19 (56%); (vi) THF, Et$_3$N, 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%.
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*.

<table>
<thead>
<tr>
<th>Compound (#)</th>
<th>Inhibition ± SD (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.10 mM</td>
</tr>
<tr>
<td>Rutalexin (5)</td>
<td>20 ± 4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Camalexin (1)</td>
<td>76 ± 0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>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 (<sup>b</sup>–<sup>e</sup>) 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](image)

**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.
(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.

![Structure of disulfide 220](image)

**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 $^1$H NMR spectrum (CD$_3$CN) displayed signals for 28 protons: five aromatic ($\delta_H$ 7–7.9 ppm), two D$_2$O exchangeable ($\delta_H$ 7.3–9.9 ppm), two vinyl ($\delta_H$ 6 and 6.3 ppm), one methyl attached to oxygen ($\delta_H$ 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 $\delta_H$ 6.37 correlated to $C_6$, $C_9$ and $C_{12}$ at $\delta_C$ 160.5, 76.8 and 26.8, respectively; $\delta_H$ 6.10 correlated to $C_3$, $C_6$ and $C_7$ at $\delta_C$ 102.5, 160.5 and 129.7, respectively; the methyl protons at $\delta_H$ 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 $^1$H and $^{13}$C NMR spectroscopic data of the unknown metabolite with $^1$H 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 $^1$H 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).

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

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).

![Figure 2.41](image)

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

^aThe 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 sulfanyl amide 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 sulfanyl amide 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) NaClO₂, 2-methylbut-2-ene, tert- butyl alcohol/H₂O, r.t., 2 h, 96%; (ii) SOCl₂, THF, rt, 3 h, then CH₃NH₂, THF, 0 °C, 20 min, 85% (based on acid 16); (iii) NaSH, DMF/H₂O, 0 °C, 2 h; (iv) NaH, THF, 0 °C, 10 min then COCl₂ (20% in toluene), -78 °C to rt, 4 h, 30% (based on 17); (v) 165- 170 °C, 30 min, 100% (Pedras et al., 2004b).](image)

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).

![Structures of brassilexin (7), 1-methylbrassilexin (165) and rapalexin A (8).](image)

**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*.

<table>
<thead>
<tr>
<th>Compound (#)</th>
<th>Inhibition ± SD (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.50 mM</td>
</tr>
<tr>
<td>Brassilexin (7)</td>
<td>100 ± 0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1-Methylbrassilexin (165)</td>
<td>100 ± 0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rapalexin A (8)</td>
<td>100 ± 0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>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.
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* <sub>R</sub> = 6.2 min), isatin (224, *t* <sub>R</sub> = 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.
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](image)

**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.
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](image)

**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 NaBH₄ in ethanol at room temperature (Scheme 2.24) (Bergonzini and Melchiorre, 2011).
Scheme 2.24 Syntheses of 3-hydroxyindoline-2-one (226) and 1-methyl-3-hydroxyindoline-2-one (227). Reagents and conditions: (i) NaBH₄, 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.

<table>
<thead>
<tr>
<th>Compound (#)</th>
<th>Inhibition ± SD (%)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.50 mM</td>
</tr>
<tr>
<td>Isatin (224)</td>
<td>63 ± 0ᵇ</td>
</tr>
<tr>
<td>Methylisatin (225)</td>
<td>32 ± 0ᵈ</td>
</tr>
<tr>
<td>3-hydroxyindoline-2-one (226)</td>
<td>46 ± 0ᶜ</td>
</tr>
<tr>
<td>3-hydroxy-1-methylindoline-2-one (227)</td>
<td>14 ± 3ᵍʰ</td>
</tr>
</tbody>
</table>

¹ 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–ᵗ) 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
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
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](image)

**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, H₂SO₄, 90 °C, 2 h, 58%.

2-(1H-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.
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 CH₃CN 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.*

<table>
<thead>
<tr>
<th>Time (Days)</th>
<th>Amount of product 26 (nmol)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amount of protein (µg in 50 µl of cell-free extracts)</th>
<th>Specific activity of BO (nmol/mg/min)&lt;sup&gt;b&lt;/sup&gt; ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dialyzed</td>
<td>Non-dialyzed</td>
<td>Dialyzed</td>
</tr>
<tr>
<td>0</td>
<td>n. d.</td>
<td>n. d.</td>
<td>70.2 ± 1.2</td>
</tr>
<tr>
<td>5</td>
<td>43.0 ± 2.0</td>
<td>n. d.</td>
<td>70.6 ± 0.90</td>
</tr>
<tr>
<td>9</td>
<td>52.0 ± 1.2</td>
<td>70 ± 20</td>
<td>94.5 ± 0.10</td>
</tr>
<tr>
<td>13</td>
<td>48.6 ± 0.80</td>
<td>72 ± 40</td>
<td>100.2 ± 0.90</td>
</tr>
<tr>
<td>15</td>
<td>54.3 ± 1.4</td>
<td>89 ± 30</td>
<td>98.0 ± 0.50</td>
</tr>
<tr>
<td>32</td>
<td>50.6 ± 0.40</td>
<td>80 ± 60</td>
<td>100.2 ± 0.70</td>
</tr>
</tbody>
</table>

<sup>a</sup> nmols of indole-3-carboxaldehyde (26) were determined using a calibration curve prepared from pure indole-3-carboxaldehyde (26).

<sup>b</sup> 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.

<sup>c</sup> 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*.

<table>
<thead>
<tr>
<th>Substrate (#) +Inhibitor (#)</th>
<th>Specific activity(^b, d) (nmol/mg/min) ± SD</th>
<th>Inhibition (%)(^c, d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.10 mM</td>
<td>0.30 mM</td>
</tr>
<tr>
<td>Brassinin (9) + Camalexin (1)</td>
<td>14.7 ± 0.9</td>
<td>9.3 ± 0.5</td>
</tr>
<tr>
<td>Brassinin (9) + 5’-methylcamalexin (174)</td>
<td>18.3 ± 0.5</td>
<td>15.1 ± 0.9</td>
</tr>
<tr>
<td>Brassinin (9) + 4-(1H-indol-3-yl)thiazole (169)</td>
<td>22.7 ± 0.3</td>
<td>19.0 ± 0.7</td>
</tr>
<tr>
<td>Brassinin (9) + 2-(1H-indol-3-yl)oxazole (167)</td>
<td>20.7 ± 0.2</td>
<td>16.6 ± 1.3</td>
</tr>
<tr>
<td>Brassinin (9) + (2-(1H-indol-3-yl)thiazol-4-yl)methanol (185)</td>
<td>21.6 ± 0.7</td>
<td>16.9 ± 0.8</td>
</tr>
<tr>
<td>Brassinin (9) + 4-(1H-indol-3-yl)isothiazole (168)</td>
<td>22.2 ± 1.0</td>
<td>14.1 ± 0.7</td>
</tr>
<tr>
<td>Brassinin (9) + 5-(1H-indol-3-yl)thiazole (170)</td>
<td>18.0 ± 0.7</td>
<td>15.5 ± 0.6</td>
</tr>
<tr>
<td>Brassinin (9) + 4’-methylcamalexin (173)</td>
<td>20.4 ± 1.5</td>
<td>20.1 ± 0.9</td>
</tr>
<tr>
<td>Brassinin (9) + 2-(1H-indol-3-yl)oxazol-4(5H)-one (229)</td>
<td>22.4 ± 0.6</td>
<td>18.1 ± 1.3</td>
</tr>
<tr>
<td>Brassinin (9) + 2-(1H-indol-3-yl)thiazole-5-carboxylic acid (184)</td>
<td>27.3 ± 1.0</td>
<td>26.3 ± 2.2</td>
</tr>
<tr>
<td>Brassinin (9) + 2-(1H-indol-3-yl)thiazole-4-carboxylic acid (230)</td>
<td>25.5 ± 0.4</td>
<td>24.1 ± 0.7</td>
</tr>
<tr>
<td>Brassinin (9) + Ethyl 2-(1H-indol-3-yl)thiazole-4-carboxylate (191)</td>
<td>23.5 ± 0.7</td>
<td>15.3 ± 1.2</td>
</tr>
</tbody>
</table>

\(^a\)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.

\(^d\)% 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-(1H-indol-3-yl)isothiazole (168, ca. 13% at 0.10 mM and 45% at 0.30 mM) and ethyl 2-(1H-indol-3-yl)thiazole-4-carboxylate (191, 8% at 0.10 mM and 40% at 0.30 mM). 2-(1H-indol-3-yl)thiazole-5-carboxylic acid (184) and 2-(1H-indol-3-yl)thiazole-4-carboxylic acid (230) (6% at 0.30 mM) did not show any activity against BO. 4-(1H-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-(1H-indol-3-yl)oxazole (167), (2-(1H-indol-3-yl)thiazol-4-yl)methanol (185) and 2-(1H-indol-3-yl)oxazol-4(5H)-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-(1H-
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
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**

- Design, synthesize and evaluate the effect of compounds derived from other potential heterocyclic skeletons (not indolyl) in the activity of BOLm.
- Investigation of transformation of rutalexin (5) by other cruciferous pathogens.
- Determine the antifungal activity of compounds 1, 167-170, 173, 174, 184, 185, 191, 229 and 230 against L. maculans.
- 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 Et₂O over sodium and benzophenone, DCM, CH₃CN, and benzene over CaH₂). Organic extracts were dried over Na₂SO₄ 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 F₂₅₄ (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) H₂SO₄, 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 ¹H NMR (500 or 600 MHz) and ¹³C NMR (125.8 MHz), the chemical shift values (δ) are reported in parts per million (ppm) relative to tetramethylsilane (TMS). For ¹H NMR (500 or 600 MHz) the δ values were referenced to CDCl₃ (CHCl₃ at 7.27 ppm), CD₃CN (CHD₂CN at 1.94 ppm), CD₃OD (CHD₂OD at 3.31) or (CD₃)₂SO (DMSO-d₅ 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 ¹³C NMR (125.8 MHz) the δ values are referenced to CDCl₃ (77.23 ppm), CD₃CN (118.69 ppm), CD₃OD (49.15 ppm), (CD₃)₂SO (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$_2$O–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$_2$O–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$_2$O–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$_2$O–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$_2$O–CH$_3$CN (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-C18 column (5 mm particle size silica, 150 × 4.6 mm I.D.). The mobile phase consisted of a linear gradient of:

**Method F:** H$_2$O (with 0.20% HCO$_2$H)-CH$_3$CN (with 0.20% HCO$_2$H) 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$_2$O (with 0.20% HCO$_2$H)-CH$_3$CN (with 0.20% HCO$_2$H) 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$_3$CN, MeOH, or mixture of H$_2$O-MeOH (50:50).

MS [high resolution (HR), electron ionization (EI)] were obtained on a VG 70 SE and Jeol AccuToF 4G GCv mass spectrometers.
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$_2$HPO$_4$ (0.75 g/l, 4.3 mM), KH$_2$PO$_4$ (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$_2$·2H$_2$O (0.10 g/l, 0.68 mM), MgSO$_4$·7H$_2$O (0.50 g/l, 2.0 mM);

**Solution 3:** ZnSO$_4$.7H$_2$O (0.40 mg/l, 1.4 µg), CuSO$_4$.5H$_2$O (0.079 mg/l, 0.32 µM), MnSO$_4$.4H$_2$O (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$_2$B$_4$O$_7$.10H$_2$O (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 °C 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-methoxycamalexin (3). Reagents and conditions: (i) Mel, Mg, Et₂O, r.t.; (ii) 2-Bromothiazole, Benzene, 24 h, 90 °C, 80% (1) and 56% (3) (Ayer et al., 1992)(Pedras et al., 2011b).](attachment:image.png)

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) $\lambda_{max}$ (nm): 215, 275, 315.  
$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 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) $\lambda_{max}$ (nm): 220, 290, 320.  
$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 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$_3$OH-H$_2$O) $\lambda_{\text{max}}$(nm): 220, 270, 330.
$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 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](image)

**Scheme 3.3** Synthesis of 4’-methylcamalexin (173). Reagent and condition: (i) CH₃COCH₂Cl, 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 H₂O. Aqueous phase was neutralized with NaHCO₃ (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, CH₃OH-H₂O) $\lambda_{\text{max}}$ (nm): 220, 280, 320.
FTIR (KBr) $\nu_{\text{max}}$ cm⁻¹: 3119, 2222, 1534, 1445, 1242, 1116, 733.
$^1$H NMR (500 MHz, CDCl₃): $\delta$ 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, CDCl₃): $\delta$ 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 C₁₂H₁₀N₂S) (100%).
3.3.1.4 5′-Methylcamalexin (174)

Scheme 3.4 Synthesis of 5′-methylcamalexin (174). Reagents and conditions: (i) Br₂, 1,4-dioxane, 1 h, 0 °C; (ii) CH₃CHBrCHO (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 NaHCO₃ (satd. sol.), and extracted with CH₂Cl₂. 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 tᵣ = 16.3 min (method A).
UV (HPLC, CH₃OH–H₂O) λₘₐₓ (nm): 220, 278, 320.
FTIR (KBr) νₘₐₓ cm⁻¹: 2833, 1540, 1451, 1242, 1119, 740.
¹H NMR (500 MHz, CDCl₃): 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$_3$): $\delta$ 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](image)

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 crystallized with EtOH. The crystals were washed with diethyl ether after filtration to afford 2-chloro-1-(1H-indol-3-yl)ethanone (177). Thioformamide (29 mg, 0.47 mmol) was added to the solution of 2-chloro-1-(1H-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-(1H-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, CH₃OH-H₂O) λ<sub>max</sub> (nm): 220, 278, 320.

FTIR (KBr) ν<sub>max</sub> cm<sup>-1</sup>: 3438, 3070, 1566, 1416, 1253, 1102, 990, 819.

<sup>1</sup>H NMR (500 MHz, CD₃CN): 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).

<sup>13</sup>C NMR (500 MHz, CD₃CN): 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]<sup>+</sup>), calcd. 200.0408 for C<sub>11</sub>H<sub>8</sub>N<sub>2</sub>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) NaN₃, H₂O, acetone, 20 h, 50 °C; (ii) Pd/C, H₂, HCl, MeOH, 3 h, r.t., 85%; (iii) CH₃CHO, Et₃N, 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 H₂O (10 ml) was heated at 50 °C for 20 h. The reaction mixture was cooled to room temperature, was diluted with H₂O (20 ml) and extracted with CH₂Cl₂. The combined extracts were dried and concentrated to dryness. The crude product was washed with CH₂Cl₂ 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 H₂-filled balloon and the reaction mixture was stirred at room temperature under H₂ atmosphere for 3 h. The reaction mixture was filtered and the filtrate was concentrated. The crude product was subjected to FCC (silica gel, MeOH-CH₂Cl₂, 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 Et₃N (50 µl). The reaction mixture was heated at 50 °C for 8 h, concentrated and diluted with H₂O. 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-CH₂Cl₂, 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-oxoethyl)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 H₂O. The aqueous solution was neutralized using NaHCO₃ (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, CH₃OH–H₂O) λ_max (nm): 210, 242, 262, 300.
FTIR (KBr) ν_max cm⁻¹: 3204, 1566, 1416, 1253, 823.
¹H NMR (500 MHz, CD₃CN): 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).
¹³C NMR (500MHz, CD₃CN): 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 C₁₁H₈N₂S) (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) POCl₃, DMF; (ii) NaOH, 1,4-dioxane; (iii) SOCl₂, THF, 10 min, -20 °C; (iv) NH₄SCN, 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 POCl₃ (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 CH₂Cl₂. 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%). SOCl₂ (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. NH₄SCN (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-(4ʹ-isothiazolyl)indole (168, 16 mg, 56% yield) as light yellowish powder.

Melting point: 124–125 °C  
HPLC tR = 12.2 min (method A).  
UV (HPLC, CH₃OH-H₂O) λmax (nm): 225, 290.  
FTIR (KBr) νmax cm⁻¹: 3406, 1582, 1457, 1243, 890.  
¹H NMR (500 MHz, CDCl₃): 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).  
¹³C NMR (125.8MHz, CDCl₃): 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 C₁₁H₈N₂S) (100%).
3.3.1.8 1H-3-(2′-Oxazolyl)indole (167)

![Scheme 3.8](image)

**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\textsubscript{3}OH-H\textsubscript{2}O) \( \lambda_{\text{max}} \) (nm): 220, 260, 297.

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

\(^1\)H NMR (500 MHz, CDCl\textsubscript{3}): \( \delta \) 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 (125.8 MHz, CDCl\textsubscript{3}): \( \delta \) 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\textsubscript{11}H\textsubscript{8}N\textsubscript{2}O) (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-thiocarboxamide (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) $\lambda_{\text{max}}$ (nm): 210, 255, 270, 350.

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

$^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 12.6 (br, 1H), 8.6 (d, $J = 3.5$ Hz, 1H), 8.2 (dd, $J = 2$, 0.5 Hz, 1H), 7.6 (dd, $J = 3.5$, 6 Hz, 1H), 7.3 (m, 2H), 4.1 (s, 2H).

$^{13}$C NMR (125.8MHz, DMSO-$d_6$): $\delta$ 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_3OH-H_2O) λ_{max} (nm): 220, 275, 325.
FTIR (KBr) ν_{max} cm^{-1}: 3247, 1710, 1540, 1460, 1327, 1227, 908, 725.
^1H 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).
^13C 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$_3$OH-H$_2$O) $\lambda_{\text{max}}$ (nm): 220, 270, 320.
FTIR (KBr) $\nu_{\text{max}}$ cm$^{-1}$: 3350, 1695, 1542, 1244, 732.
$^1$H NMR (500 MHz, MeOD): $\delta$ 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): $\delta$ 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$_8$N$_2$O$_2$S) (100%).

3.3.2 Brassinin (9)

![Scheme 3.11](image)

**Scheme 3.11** Synthesis of brassinin (9). Reagents and conditions: (i) NH$_2$OH.HCl, Na$_2$CO$_3$, 1 h, 80 °C, 95%; (ii) NiCl$_2$.6H$_2$O, NaBH$_4$, MeOH, 10 min, 0 °C, 70%; (iii) Et$_3$N, pyridine, CS$_2$, 10 min, 0 °C; (iv) MeI, 30 min, 0 °C, 73% (Sharma-Mamillapalle, 2012).

A solution of NH$_2$OH.HCl (957 mg, 13.8 mmol) and Na$_2$CO$_3$ (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-carboxaldehyde (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-carboxaldehyde (27, 700 mg, 4.40 mmol) and NiCl$_2$.6H$_2$O (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$_4$OH (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$_4$OH, 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$_3$OH-H$_2$O) $\lambda_{\text{max}}$(nm): 217, 270.
$^1$H NMR (500 MHz, CD$_3$CN): $\delta$ 9.25 (br, 1H, D$_2$O exchangeable), 8.24 (s, 1H, D$_2$O 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, CH₂Cl₂-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, CH₃OH-H₂O) $\lambda_{\text{max}}$(nm): 210, 280.
$^1$H NMR (500 MHz, CDCl₃): 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](image)

**Scheme 3.13** Synthesis of 1-methylcyclobrassinin (193). Reagents and conditions: (i) NaH, MeI, THF, 1 h, 0 °C, 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) \( \lambda_{\text{max}} \) (nm): 206, 230, 300.

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

3.3.4 Rutalexin (5)

![Scheme 3.14](image)

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).
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 MeNH₂ 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<sub>R</sub> = 11.6 min (method B).
UV (HPLC, CH₃OH-H₂O) λ<sub>max</sub> (nm): 210, 280.
¹H NMR (500 MHz, CD₃OD): 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).
¹³C NMR (500 MHz, CD₃OD): 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 C₂₄H₂₈N₂O₅S).

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) $\lambda_{\text{max}}$ (nm): 213, 242, 275.
$^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 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-$d_6$): $\delta$ 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 \( t_R = 21.3 \text{ min (method B)} \).

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

\(^1\)H NMR (500 MHz, \( \text{CD}_3\text{CN} \)): \( \delta \) 8.30 (d, \( J = 6.5, 1\text{H} \)), 8.11 (d, \( J = 7.5, 1\text{H} \)), 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 \( \times 2 \)) 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 \( t_R = 8.5 \text{ min (method B)} \).

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

\(^1\)H NMR (500 MHz, DMSO-\( d_6 \)): \( \delta \) 12.57 (br, 1H, \( D_2\text{O} \) exchangeable), 8.09 (m, 1H), 7.54 (m, 1H), 7.28 (m, 2H), 3.37 (s, 3H).

\(^{13}\)C NMR (125 MHz, DMSO-\( d_6 \)): \( \delta \) 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](image)

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) $\lambda_{max}$ (nm): 220, 250, 265.

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 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 °C, 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 °C. The reaction mixture was stirred for 15 minutes at 0 °C followed by addition of methyl iodide (27 µl, 0.44 mmol). The reaction mixture was stirred for an additional 1 h at 0 °C. 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, 0.30 mmol, 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) $\lambda_{\text{max}}$ (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$_3$CN) 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$_3$CN (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$_2$O, mycelia of cultures were filtered off after 48 h of incubation, were washed with water and transferred into sterile H$_2$O; a solution of the compound to be tested (dissolved in CH$_3$CN) 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$_3$CN or MeOH–H$_2$O (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$_3$CN) 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$_3$CSNH$_2$, 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$_3$OH-H$_2$O) $\lambda_{\text{max}}$ (nm): 215, 255, 318.

FTIR (KBr, cm$^{-1}$) $\nu_{\text{max}}$: 3190, 1621, 1527, 1442, 850.

$^1$H NMR (DMSO-$d_6$) $\delta$: 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-$d_6$) $\delta$: 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$_9$H$_8$N$_2$S) (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) $\lambda_{\text{max}}$ (nm): 220, 260, 330.
$^1$H NMR (500 MHz, CD$_3$CN): $\delta$ 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): $\delta$ 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](image)

**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 Ac₂O (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 \( t_R = 7.9 \text{ min} \) (method A).
UV (HPLC, CH₃OH-H₂O) \( \lambda_{\text{max}} \) (nm): 220, 270, 290.
FTIR (KBr) \( \nu_{\text{max}} \) cm⁻¹: 3383, 1612, 1451, 1122, 739.
\(^1\)H NMR (500 MHz, CD₃CN): \( \delta \) 8.50 (br, 1H), 7.60 (s, 1H), 7.63 (m, 2H), 6.97 (dd, \( J = 8.5, 2 \text{ Hz} \) 1H), 6.93 (s, 1H), 3.91 (s, 3H).
\(^{13}\)C NMR (500MHz, CD₃CN): \( \delta \) 157.6, 136.2, 132.4, 120.8, 119.4, 115.9, 112.2, 95.4, 85.4, 55.2.
HRMS-ESI \( m/z \): measured 172.0638 ([M]⁺, calcd. 172.0637 for C₁₀H₈N₂O) (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 NaHCO₃ (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 \( t_R = 4.5 \text{ min} \) (method A).
UV (HPLC, CH₃OH-H₂O) \( \lambda_{\text{max}} \) (nm): 220, 250, 280.
FTIR (KBr) \( \nu_{\text{max}} \) cm⁻¹: 3399, 3250, 1630, 1530, 1040.
\(^1\)H NMR (500 MHz, CD₃CN): \( \delta \) 9.68 (br, 1H), 8.24 (d, \( J = 8.5 \text{ Hz} \), 1H), 7.83 (d, \( J = 2.5 \text{ Hz} \), 1H), 7.60 (br, 2H), 7.00 (d, \( J = 2.5 \text{ Hz} \), 1H), 6.86 (dd, \( J = 8.5, 2.5 \text{ Hz} \), 1H), 3.81 (s, 3H).
\(^{13}\)C NMR (500 MHz, CD₃CN): \( \delta \) 195.3, 156.7, 137.9, 128.0, 121.9, 119.25, 111.4, 100.0, 95.1, 55.1.
HRMS-ESI \( m/z \): measured 206.0515 ([M]⁺, calcd. 206.0514 for C₁₀H₁₀N₂OS) (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, Mel, 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$_3$OH-H$_2$O) $\lambda_{max}$ (nm): 220, 280.
FTIR: 3331, 3116, 2932, 2223, 1528, 1459, 1383, 1335, 1254, 1195, 1136 cm$^{-1}$.
$^1$H NMR (500 MHz, CD$_3$OD): $\delta$ 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$_3$CN): $\delta$ 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$_8$N$_2$) (100%).
3.4.1.3.4 1-Methylindole-3-carboxylic acid (94) and 6-methoxyindole-3-carboxylic acid (95)

Scheme 3.21 Syntheses of 1-methylindole-3-carboxylic acid (94) and 6-methoxyindole-3-carboxylic acid (95). Reagents and conditions: (i) NaClO₂, NaH₂PO₄, t-butanol, 2-methylbut-2-ene, H₂O, r.t., 14 h, 75% (94) and 60 h, 52% (95).

A solution of NaClO₂ (25 eq) and NaH₂PO₄ (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 tᵣ = 4.7 min (method A).
UV (HPLC, CH₃OH-H₂O) λₘₐₓ (nm): 215, 290.
¹H 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).
¹³C 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 C₁₀H₉N₁O₂) (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) $\lambda_{\text{max}}$ (nm): 215, 275.
$^1$H NMR (500 MHz, MeOD): $\delta$ 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): $\delta$ 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) $\lambda_{\text{max}}$ (nm): 220, 280.

$^1$H NMR (500 MHz, MeOD): $\delta$ 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): $\delta$ 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)

\[ 
\begin{array}{c}
\text{N} & \text{S} \\
\text{N} & \text{S} & \text{COOEt} \\
\text{i} & \rightarrow & \text{N} & \text{OH} \\
\text{191} & \rightarrow & \text{185} \\
\end{array} 
\]

**Scheme 3.23** Synthesis of 2–1$H$-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 (2–1$H$-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) $\lambda_{\text{max}}$ (nm): 220, 280, 320.

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

$^1$H NMR (500 MHz, CD$_3$OD): $\delta$ 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): $\delta$ 164.6, 156.3, 136.9, 125.2, 124.4, 122.3, 120.5, 119.4, 111.4, 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](image)

Scheme 3.24 Synthesis of 2-(1H-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-(1H-indol-3-yl)thiazole-5-carboxylate (45 mg, 62%). NaOH (25% aq, w/v, 0.30 ml) was added to a solution of 2-(1H-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-(1H-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) $\lambda_{\text{max}}$ (nm): 220, 280, 340.

FTIR (KBr) $\nu_{\text{max}}$ cm$^{-1}$: 3199, 2392, 1637, 1526, 1220, 1081, 826.
\(^1\)H NMR (500 MHz, CD\(_3\)OD): \(\delta\) 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): \(\delta\) 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-El \(m/z\): 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 \(\times\) 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, CH₃OH-H₂O) $\lambda_{max}$ (nm): 218, 335(br).
FTIR (KBr) $\nu_{max}$ cm⁻¹: 3408, 3319, 2924, 1644, 1621, 1507, 1211.
$^1$H NMR (500 MHz, DMSO-d₆): $\delta$ 11.57 (br, 1H), 8.22 (brt, $J = 2.5$ Hz, 1H), 7.56 (d, $J = 7.5$ Hz, 1H), 7.37 (d, $J = 7.5$ Hz, 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$_6$): $\delta$ 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$_2$O$_2$). 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) $\lambda_{max}$ (nm): 220, 345.
FTIR (KBr) $v_{max}$ cm$^{-1}$: 3318, 2929, 1640, 1524, 1459, 1370, 1308, 1217.
$^1$H NMR (500 MHz, DMSO-d$_6$): $\delta$ 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$_6$): $\delta$ 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$_2$O$_2$). 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) $\lambda_{max}$ (nm): 238, 308.
FTIR (KBr) $v_{max}$ cm$^{-1}$: 3391, 2922, 2852, 1703, 1618, 1496, 1464, 1450, 1348, 1249, 1226, 1140, 1016.
$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 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](image)

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-\textit{d$_6$}): $\delta$ 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](image)

**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'-thiopyran]-2-[3,2-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) $\lambda_{max}$ (nm): 237, 297, 330.
FTIR (KBr) $\nu_{\text{max}}$ cm$^{-1}$: 3048, 2924, 1720, 1610, 1464, 1369, 1308, 1186, 1162, 1095.

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 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 (dd, $J$ = 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$_3$): $\delta$ 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-ESI $m/z$: 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) $\lambda_{\text{max}}$ (nm): 237, 300.

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

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 7.36 (dd, $J$ = 7.5, 7.5 Hz, 1H), 7.32 (d, $J$ = 7.5 Hz, 1H), 7.30 (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), 3.78 (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$_3$): $\delta$ 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-ESI $m/z$: 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) $\lambda_{\text{max}}$ (nm): 235, 305.

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

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 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$_3$): $\delta$ 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'-thiopyran[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) $\lambda_{\text{max}}$ (nm): 235, 300, 340.

FTIR (KBr) $v_{\text{max}}$ cm$^{-1}$: 2926, 1620, 1460,1379.

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 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$_3$): $\delta$ 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](image)

**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, CHCl₃-hexanes, 7:3) to afford S-methyl [(2-sulfanyl methyl-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-sulfanyl methyl-1Hindolyl-3)methyl] carbamothioate (214, 10 mg, 0.038 mmol, 63%).

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

Brownish gum.

HPLC $t_R = 11.3$ min (method A).

UV (HPLC, CH₃OH-H₂O) $\lambda_{max}$ (nm): 285, 220.

FTIR (KBr) $\nu_{max}$ cm⁻¹: 3290, 2927, 2359, 1650, 1505, 1450, 1311, 740.

¹H NMR (500 MHz, CDCl₃): $\delta$ 8.19 (br, 1H), 7.63 (d, $J=5$ Hz, 1H), 7.34 (d, $J= 5$Hz, 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 \textit{m/z}: measured 266.0539 ([M$^+$], calcd. 266.0548 for C$_{12}$H$_{14}$N$_2$O$_2$S$_2$). MS (EI) \textit{m/z} (% relative int.): 266.05 (53), 219.05 (55), 176.05 (100), 117.05 (21).

\textit{S-methyl [(1-methyl-2-sulfanylmethyl-1Hindolyl-3)methyl] carbamothioate} (214)

Yellowish powder.

Melting point: 146–150 °C.

HPLC \textit{t}_R = 15.9 min (method A).

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

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

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 7.66 (d, \textit{J} = 5 Hz, 1H), 7.31 (m, 1H), 7.17 (dd, \textit{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 \textit{m/z}: measured 280.0716 ([M$^+$], calcd. 280.0714 for C$_{13}$H$_{16}$N$_2$O$_2$S$_2$). MS (EI) \textit{m/z} (% relative int.): 280.07 (38), 233.08 (70), 190.07 (100).

\subsection*{3.4.3 Rutalexin}

\subsubsection*{3.4.3.1 Biotransformation of rutalexin}

Time course experiment for rutalexin (5) was carried out in MM. Cultures of \textit{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 $\times$ 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).

\[
\text{HPLC } t_R = 9.9 \text{ min (method B).}
\]

UV (HPLC, CH$_3$OH-H$_2$O) $\lambda_{\text{max}}$ (nm): 220, 300.

FTIR (KBr) $\nu_{\text{max}}$ cm$^{-1}$: 1680, 1627, 1551, 1463, 1384, 1226, 1171, 1013, 751.

$^1$H NMR (600 MHz, CH$_3$CN): $\delta$ 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): $\delta$ 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, H₂O, 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 \( t_R = 10.5 \text{ min} \) (method B).
UV (HPLC, CH₃OH-H₂O) \( \lambda_{\text{max}} \) (nm): 220, 310.
FTIR (KBr) $\nu_{\text{max}}$ cm$^{-1}$: 1614, 1535, 1438, 1409, 1209, 1026, 742.

$^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 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-$d_6$): $\delta$ 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$_4$O$_2$S$_2$) (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) $\lambda_{\text{max}}$ (nm): 220, 280.

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

$^1$H NMR (500 MHz, DMSO): $\delta$ 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): $\delta$ 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$_2$O$_4$S) (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)-1H-indole-1-carboxylate (217, 24 mg, in 97%) as a light yellowish powder.

HPLC $t_R = 13.4$ min (method B).
UV (HPLC, CH$_3$OH-H$_2$O) $\lambda_{\text{max}}$ (nm): 212, 293.
FTIR (KBr) $\nu_{\text{max}}$ cm$^{-1}$: 1736, 1639, 1446, 1370, 1154, 1101, 748.
$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 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$_3$): $\delta$ 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)-1H-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)-1H-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$_3$OH-H$_2$O) $\lambda_{\text{max}}$ (nm): 220, 300.
FTIR (KBr) $\nu_{\text{max}}$ cm$^{-1}$: 2926, 1725, 1618, 1543, 1413, 1313, 1148, 750.
$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 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$_3$): $\delta$ 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

\[
\begin{align*}
\text{Isatin (224, 147 mg, 1.00 mmol) was added to a solution of NaBH}_4 (111 mg, 3.00 mmol) \\
in \text{EtOH (95%, 10 ml). The reaction mixture was stirred at room temperature for 20 min. The} \\
\text{resulting suspension was purred into cold water (30 ml) in an ice bath, acidified (HCl, pH ~ 5) } \\
\text{and extracted with chloroform. The combined extracts were dried and concentrated to dryness. The} \\
\text{residue was subjected to FCC (silica gel, DCM-MeOH, 98:2.0) to afford 226 (100 mg, 68%) as} \\
yellow powder. Similarly compound 227 was synthesized using 1-Methylisatin (161 mg, 1.00}
\end{align*}
\]
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) $\lambda_{max}$ (nm): 210, 260, 300.
FTIR (KBr) $\nu_{max}$ cm$^{-1}$: 1717, 1623, 1469, 1266, 1215, 743.
$^1$H NMR (500 MHz, MeOD): $\delta$ 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): $\delta$ 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) $\lambda_{max}$ (nm): 210, 260, 300.
FTIR (KBr) $\nu_{max}$ cm$^{-1}$: 3293, 1702, 1619, 1469, 1385, 1269, 1090, 758.
$^1$H NMR (500 MHz, CD$_3$Cl): $\delta$ 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): $\delta$ 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 CH₃CN (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 (µmol·min⁻¹ = U).
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