BIOTRANSFORMATION OF CRUCIFEROUS PHYTOALEXINS BY PATHOGENIC FUNGI

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
by
Francis Inyangala Okanga
Spring, 1999

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0-612-37905-1
UNIVERSITY OF SASKATCHEWAN
College of Graduate Studies and Research

SUMMARY OF DISSERTATION
Submitted in partial fulfillment
of the requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY
by
Francis Inyangala Okanga
Department of Chemistry
University of Saskatchewan
Spring 1999

Examinining Committee:

Dr. N.H. Low
Dean's Designate, Chair College of Graduate Studies and Research

Dr. R. E. Verrall
Chair of Advisory Committee, Department of Chemistry

Dr. M. S. C. Pedras
Supervisor, Department of Chemistry

Dr. M. Majewski
Department of Chemistry

Dr. D. E. Ward
Department of Chemistry

Dr. G. A. Hill
Department of Chemical Engineering

External Examiner:

Dr. W. A. Ayer
Department of Chemistry
University of Alberta
Edmonton, Alberta
T6G 2G2
BIOTRANSFORMATION OF CRUCIFEROUS PHYTOALEXINS BY PATHOGENIC FUNGI

Phytoalexins are part of the induced plant defenses produced in response to pathogen attack and diverse forms of stress. The biotransformation and detoxification of phytoalexins is a means that pathogens have evolved to overcome the plants defenses.

Studies on the biotransformation of the cruciferous phytoalexin cyclobrassinin, phytoalexin analogues methyl tryptamine dithiocarbamate, methyl tryptamine carbamate, N₅-carbomethoxyindole-3-methanamine and cyclobrassinin homologue by the cruciferous phytopathogenic fungi Phoma lingam, Alternaria brassicae and Rhizoctonia solani were undertaken. These studies were aimed at understanding the mechanism of phytoalexin detoxification by the pathogens. After an optimum period of incubation of the fungus and phytoalexin / analogue, cultures were filtered, the broth extracted and analyzed by TLC and HPLC. Flash column chromatography and / or preparative TLC resulted in isolation of the metabolites which were characterized by spectroscopic techniques (¹H NMR, ¹³C NMR, FTIR and MS).

Incubation of cyclobrassinin with P. lingam yielded dioxibrassinin, 3-methylenaminoinoindole-2-thione and brassilexin. Cyclobrassinin was metabolized by R. solani to yield three products identified as 2-mercaptoindole-3-carboxaldehyde, brassicanal A and 5-hydroxybrassicanal A. The biotransformation of methyl tryptamine dithiocarbamate by P. lingam yielded N₅-acetyltryptamine, indole-3-acetic acid, indole-3-carboxylic acid, methyl indole-3-acetate, methyl 2-oxotryptamine dithiocarbamate, methyl tryptamine dithiocarbamate-S-oxide oxindole-3-acetic acid, 1-thiomethylthiocarbonyl-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indol-3a-ol, tryptamine and tryptophol. A. brassicae biotransformed methyl tryptamine dithiocarbamate to yield two products identified as N₅-acetyltryptamine and tryptamine. Incubation of methyl tryptamine carbamate with P. lingam resulted in the isolation of indole-3-acetic acid, indole-3-carboxylic acid and tryptophol. Cyclobrassinin homologue yielded one
metabolite identified as cyclobrassinin homologue metabolite on incubation with *P. lingam* isolates.

For each compound and fungal isolate, further biotransformations of metabolites were carried out in order to map out the sequence and hence the biotransformation pathway(s). Bioassays were conducted to compare the antifungal activity of the parent compound and its metabolites. The results of the biotransformation studies showed that the fungal transformations of phytoalexins / analogues were detoxification processes. Cyclobrassinin was detoxified via other phytoalexins suggesting that fungal pathogens may utilize pathways that exist in plants to detoxify phytoalexins.

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<tr>
<td>December, 1979</td>
<td>B.Sc. (Hon.), Chemistry and Mathematics, University of Nairobi, Kenya</td>
</tr>
<tr>
<td>May 1984 - May 1989</td>
<td>Research Officer (Chemist), Kenya Industrial Research and Development Institute</td>
</tr>
<tr>
<td>November, 1987</td>
<td>M.Sc., Chemistry, University of Calgary, Alberta, Canada</td>
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<tr>
<td>June 1989 - current</td>
<td>Lecturer, Egerton University, Njoro, Kenya</td>
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**HONOURS**

The Agricultural Society of Kenya, young farmers clubs Sir Alec’s trophy, July 1973


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Department of Chemistry  
University of Saskatchewan  
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ABSTRACT

Studies on the biotransformation of the cruciferous phytoalexin cyclobrassinin, phytoalexin analogues methyl tryptamine dithiocarbamate, methyl tryptamine carbamate, $N_b$-carbomethoxyindole-3-methanamine and cyclobrassinin homologue by the cruciferous phytopathogenic fungi *Phoma lingam*, *Alternaria brassicae* and *Rhizoctonia solani* were undertaken. These studies were aimed at understanding the mechanism of phytoalexin detoxification by the pathogens. After an optimum period of incubation of the fungus and phytoalexin / analogue, cultures were filtered, the broth extracted and analyzed by TLC and HPLC. Flash column chromatography and / or preparative TLC resulted in isolation of the metabolites which were characterized by spectroscopic techniques ($^1$H NMR, $^{13}$C NMR, FTIR and MS).

Incubation of cyclobrassinin with *P. lingam* isolates yielded dioxibrassinin, 3-methylenaminoindole-2-thione and brassilexin as metabolites. Cyclobrassinin was metabolized by *R. solani* to yield three products identified as 2-mercaptoindole-3-carboxaldehyde, brassicanal A and 5-hydroxybrassicanal A. The biotransformation of methyl tryptamine dithiocarbamate by *P. lingam* isolates yielded $N_b$-acetyltryptamine, indole-3-acetic acid, indole-3-carboxylic acid, methyl indole-3-acetate, methyl 2-oxotryptamine dithiocarbamate, methyl tryptamine dithiocarbamate-S-oxide oxindole-3-acetic acid, methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate, tryptamine and tryptophol. The fungal pathogen *A. brassicae* biotransformed methyl tryptamine dithiocarbamate to yield two products identified as $N_b$-acetyltryptamine and tryptamine. Incubation of methyl tryptamine carbamate with *P. lingam* isolates gave indole-3-acetic acid, indole-3-carboxylic acid and tryptophol as metabolites. Cyclobrassinin homologue yielded one metabolite identified as cyclobrassinin homologue metabolite on incubation with *P. lingam* isolates.
For each compound and fungal isolate, further biotransformations of metabolites were carried out in order to map out the sequence and hence the biotransformation pathway(s). Bioassays were conducted to compare the antifungal activity of the parent compound and its metabolites. The results of the biotransformation studies showed that the fungal transformations of phytoalexins / analogues were detoxification processes.
ACKNOWLEDGEMENTS

I am grateful to my supervisor Prof. M. Soledade C. Pedras for her excellent supervision and inspiration through this project. I would like to thank my colleagues for their support; Dr. Abdul Q. Khan, John L. Sorensen, Dr. Kevin C. Smith, Irina L. Zaharia, Irving Ramirez, Claudia Erosa, Corinne J. Biesenthal, Dr. Farooq Biabani, Dr. Ali Loukaci, Corwin M. Nycholat.

I wish to express my gratitude to the members of advisory committee; Dr. D. E. Ward, Dr. M. Majewski, Dr. R. E. Verrall, Dr. G. A. Hill for their guidance. I would also like to thank my external examiner Dr. W. A. Ayer. Thanks to all those members of the Chemistry Department who made my stay in Saskatoon fruitful. Special mention is given to Dr. K. Brown and Mr. K. Thoms for their help with instrumental analysis. I am also grateful to Egerton University, Njoro, Kenya for granting me study leave and to my wife Tecla and three daughters, Helen, Josephine and Rose for being so patient. The financial support of the University of Saskatchewan (University Scholarship) and NSERC Canada is gratefully acknowledged.
To my first teachers,

my parents,

Gladys and James Okanga
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CHAPTER 1 : INTRODUCTION

1.1 General objectives

This thesis describes the results of the biotransformations of cruciferous phytoalexins and analogues. The objectives of this study were to investigate the metabolism and detoxification of cruciferous phytoalexins and analogues by pathogenic fungi. These investigations are important both in understanding the mechanism of phytoalexin detoxification by plant pathogens and in the design of selective fungicides structurally similar to phytoalexins. The biotransformation of phytoalexin analogues will enable the determination of the effect of structural modifications on the fungal response as compared to the cruciferous phytoalexins. The results obtained from these biotransformation experiments will lead to a better understanding of the role and fate of phytoalexins in the plant-pathogen interactions. This could provide leads toward understanding the part played by phytoalexins in plant susceptibility or resistance to fungal diseases.

Biotransformation studies were undertaken on:

(1) The cruciferous phytoalexin cyclobrassinin (2).

(2) Methyl tryptamine dithiocarbamate (3), a homologue of the phytoalexin brassinin (1).

(3) Phytoalexin analogues methyl tryptamine carbamate (4), N\textsubscript{6}-carbomethoxyindole-3-methanamine (5) and cyclobrassinin homologue (6).

The structures of these compounds are shown in Figure 1.1. The fungal pathogens employed in these biotransformation studies were the blackleg fungus [Leptosphaeria maculans (Desm.) Ces. et de Not., asexual stage Phoma lingam (Tode ex Fr.) Desm.],
*Rhizoctonia solani* Kuhn [teleomorph: *Thanatephorus cucumeris* (Frank) Donk] and *Alternaria brassicae* (Berk.) Sacc.

![Molecular structures](image)

**Figure 1.1** Structures of brassinin (1), cyclobrassinin (2), methyl tryptamine dithiocarbamate, (3), methyl tryptamine carbamate (4), N₆-carbomethoxyindole-3-methanamine (5) and cyclobrassinin homologue (6).

### 1.2 Cruciferous crops

Cruciferous crops are cultivated worldwide and constitute an extremely valuable group of plants. The tribe *Brassicaceae* is one of the 13-19 tribes which have been recognized by diverse authors within the Crucifer family (Gómez-Campo, 1980). By far, it is the most important from an economic point of view, since it includes the genus *Brassica* with several crop species providing edible roots, stems, leaves, buds, flowers, or seeds. Brassicas include familiar vegetables such as broccoli, cabbage, cauliflower, kale and turnip. The genera *Raphanus* and *Sinapis* follow in importance, the former being
cultivated for its edible roots and the latter as a source of mustard condiments. Rapeseed is one of the major global sources of vegetable oil, which comes from several *Brassica* species belonging to the family *Cruciferae* (Lühs and Friedt, 1994). These oil crop species include *B. napus, B. rapa* (syn. *B. campestris*) and *B. juncea*. Brassica oilseed crops have become the third most important world source of vegetable oil after soybean and palm. Furthermore, rapeseed also ranks third in the production of oilseed meal after soybean and cotton (Lühs and Friedt, 1994).

In brassica oilseeds, the occurrence of two components traditionally distinguished them from other major oilseeds (Lühs and Friedt, 1994). Both components, erucic acid and glucosinolates, were considered anti-nutritional for humans and for animals. Plant breeders successfully altered the chemical composition of rapeseed and introduced oils virtually free of erucic acid. This enhanced its attractiveness as an edible oil. The breeding of low erucic acid rapeseed cultivars with very low glucosinolate content in the meal additionally increased its potential as a protein supplement in animal feeds. Currently, in the major rapeseed producing areas (Canada, Europe) production has been shifted almost completely to *B. napus* and *B. rapa* cultivars with minor quantities of both erucic acid and glucosinolates in the oil and the meal, respectively. In Canada this double-low seed type is called "canola" to facilitate identification of the seed and the products (oil and meal) made from it. Canola is currently defined as having less than 2% erucic acid in the oil and less than 30 micromoles of the aliphatic glucosinolates per gram of oil-free meal. Canola oil is utilized primarily for food purposes as margarine, shortening, cooking and salad oil. In Canada, canola and rapeseed are important agricultural crops, especially in the prairie provinces of Manitoba, Saskatchewan and Alberta. Canola oil accounts for about 60% of the domestic production of refined oil (Lühs and Friedt, 1994). Most rapeseed producing countries utilize the crop internally. Of those that export, Canada and the European Union share about 90% of world trade almost equally between them (Kimber and McGregor, 1995).
1.3 Fungal pathogens of crucifers

1.3.1 Phoma lingam

The blackleg fungus [Leptosphaeria maculans (Desm.) Ces. et de Not., asexual stage phoma lingam (Tode ex Fr.) Desm.] is the causal agent of blackleg (stem canker) of cruciferous plants. Blackleg is a serious disease of crucifers in many parts of the world (Keri et al., 1997; Mayerhofer et al., 1997). In Canada, losses to the canola industry caused by blackleg infestation can exceed 50% and result in up to $100 million annually of lost revenue (Meyerhofer et al., 1997).

Phoma lingam attacks cotyledons, leaves, stems and roots of crucifers (Gugel and Petrie, 1992). Infection of seed pods and seed may occur on crucifer seed crops. Lesions caused by the fungus on cotyledons and leaves are generally greyish to dirty white in color and vary in size and shape. Numerous pinhead-sized, black asexual fruiting bodies called pycnidia are often present in the dead tissue at the centres of these lesions. The blackleg fungus can be differentiated into two types of isolates, virulent and avirulent (Williams, 1992). The virulent type of P. lingam causes sunken, cracked stem cankers that can girdle and extend up the stem. Stem cankers result in decreased nutrient flow and / or lodging of the plants, both of which can cause a decrease in crop yield (Gugel and Petrie, 1992). Stem infection by the virulent isolate of P. lingam before the six-leaf stage is associated with severe losses in yield. Lesions caused by the avirulent isolate generally become apparent later in the growing season, at about the time of plant senescence; these lesions tend to be small and shallow, causing only slight injury to plants. Recently, a general view has been expressed that virulent and avirulent isolates belong to different species (Taylor et al., 1991; Koch et al., 1991; Morales et al., 1993 a, b; Williams, 1992); however, no formal reclassification has been done (Pedras et al., 1996).

Phoma lingam has various modes of survival incorporated into its life cycle (Williams, 1992). Primary dispersal occurs through colonized crucifer seed and crop
residue. Both pycnidia and pseudothecia survive and under suitable climatic conditions produce rain splash-dispersed pycnidiospores and airborne ascospores. Pycnidiospores and germ tubes of ascospores gain entry to host tissues through stomata or wounds and hyphae grow intercellularly with the plant cells. The fungus then induces cell death and degradation causing necrotic leaf lesions or stem cankers and produces pycnidia with copious pycnidiospores. The pycnidiospores constitute secondary inoculum and are dispersed by rainsplash and wind to neighbouring plants (Gugel and Petrie, 1992).

Like other plant pathogens virulent isolates of the blackleg fungus produce phytoxins. *P. lingam* produces the host-selective toxin phomalide (7) (Pedras et al., 1993; Pedras, 1997; Ward et al., 1996) together with a complex mixture of nonselective phytoxins [epipolythiodioxopiperazines (EPTs)] such as sirodesmin PL (8) (Figure 1.2) (Férezou et al., 1977; Pedras et al., 1988, 1989, 1990, Pedras and Séguin-Swartz, 1992). Phomalide causes lesions on canola (blackleg susceptible cultivar) that closely resemble the symptomatic leaf lesions caused by the blackleg disease. By contrast, mustard (*B. juncea*, blackleg resistant cultivar) is only slightly sensitive to phomalide (Pedras et al., 1993, 1996).

![Figure 1.2 Structures of phomalide (7) and sirodesmin PL (8)](image-url)
Studies on the secondary metabolite profile of avirulent isolates have shown (Pedras et al., 1995, 1996) an intriguing relationship between Phoma lingam and Phoma wasabiae, also a pathogen of crucifers. P. wasabiae is the causative agent of blackleg disease of the crucifer wasabi or Japanese horseradish (Eutrema wasabiae M.). The metabolites obtained from P. wasabiae were identical in all respects to the metabolites isolated from P. lingam. This relationship was further supported by molecular genetic characteristics and pathogenicity tests (Pedras et al., 1996). P. wasabiae and avirulent isolates of P. lingam cause similar disease symptoms on wasabi (E. wasabi M.) plants. These findings provide evidence that avirulent isolates of P. lingam are chemically and genetically similar to P. wasabiae. It is likely that P. wasabiae and avirulent isolates of P. lingam are members of the same species (Pedras et al., 1996).

The blackleg disease has been controlled primarily through the use of treated seed, crop rotation and more recently moderately resistant cultivars (Keri et al., 1997). B. juncea (L.) Czern (oilseed mustard) is more resistant to P. lingam at the seedling and adult plant stages than are B. rapa (turnip oilseed rape) and B. napus (oilseed rape), and this resistance is thought to be controlled by genes located on the b-genome. There is considerable interest in Western Canada to develop canola-quality B. juncea, because of its resistance to blackleg and its superior draught tolerance. However, recently a new group of P. lingam isolates pathogenic on B. juncea has been reported (Taylor et al., 1995). Low glucosinolate B. juncea has been developed and this is an achievement that should allow the breeding of canola quality oilseed B. juncea (Love et al., 1990).

1.3.2 *Rhizoctonia solani*

*Rhizoctonia solani* Kuhn [teleomorph: Thanatephorus cucumeris (Frank) Donk], an important plant pathogen with a wide host range, is a species complex varying in cultural morphology and pathogenicity (Hyakumachi et al., 1998). Based on hyphal anastomosis
behaviour, isolates of *R. solani* have been divided into 12 anastomosis groups (AGs) denoted AG1-11 and BI. *R. solani* AG2 was divided into three subgroups, AG2-1, AG2-2 and AG2-3, on anastomosis frequency and thiamine requirement. Two cultural types within AG2-2 were distinguished by pathogenicity and cultural morphology, and placed in separate intraspecific groups (ISGs) named IIIB and IV. The main criterion for differentiating these cultural types is their relative growth in response to high temperature; IIIB isolates can grow at 35 degrees C, while IV isolates cannot.

Diseases caused by *R. solani* occur wherever cruciferous crops are grown (Howard et al., 1994). Depending on the time of infection, this fungus can cause different diseases, such as damping-off, wirestem, bottom rot, head rot and root rot. Pre- and post-emergence seedling damping-off, root rot, and basal stem rot or foot rot of adult plants are important diseases of oilseed rape and canola in Western Canada (Kataria and Verma, 1992). Root rots occur across the Canadian prairies, varying in severity in the different provinces. In Manitoba and Saskatchewan, root rot frequency has been generally low. In Alberta, however, root rot has been recognized as a serious disease. In the Peace River region in Alberta, most fields are affected to some extent each year, and sometimes, 80-100% of the plants are infected, with partial or nearly complete loss of plant stands. The fungus causes light brown lesions on the taproot and lateral roots. Lesions become sunken, and darken, and may enlarge to girdle the taproot, resulting in premature death of the plants.

The population of *R. solani* that infects oilseed rape and canola is mainly composed of anastomosis groups AG2-1 and AG4 (Kataria and Verma, 1992). Extensive surveys have been conducted in oilseed rape/canola fields in Western Canada, of the incidence and severity of *R. solani* damping-off and root rot (Kataria and Verma, 1992). In these surveys, the frequency of isolating AG2-1 was higher from seedlings than from mature canola plants; the latter yielded more AG4 isolates. Pathogenicity tests have been conducted on oilseed rape and canola cultivars (mainly *B. napus* cv. Westar and *B.*
campestris cv. Tobin) in growth chambers with various R. solani isolates recovered from canola plants and field soils in Saskatchewan and Alberta (Kataria and Verma, 1992). Generally, the isolates AG2-1 are more virulent than isolates AG4. The virulence of isolates was influenced by their source: isolates from seedlings and adult plants were more virulent than isolates of the same AG from soil; seedling isolates of AG2-1 and AG4 were more virulent than isolates from adult plants.

The fungus overwinters as mycelium and as sclerotia in soil and plant residues (Howard et al., 1994). It may be carried on or in seed. It is disseminated in infested soil or infected debris by wind, rain, irrigation water and machinery. R. solani can infect plants directly through the cuticle or through wounds or natural openings. Seedlings and young plants are more susceptible to infection than older plants (Kataria and Verma, 1992). Seedling infection by AG2-1 is favored by cool weather whereas warm weather is conducive to severe damping-off by AG4. The pathogen infects seedling hypocotyls and roots by dome-shaped infection cushions, and macerates the cortical and also the vascular tissues by cell-wall degrading enzymes. Currently grown cultivars of oilseed rape and canola are susceptible to both AG2-1 and AG4. S. alba, B. juncea, B. nigra and older plants of B. napus and B. rapa are less severely infected owing to thicker cuticles and epidermal cell walls.

Due to the lack of adequate genetic resistance in oilseed rape and canola, and the absence of practical methods for suppressing R. solani populations in the field, seed treatment with chemical fungicides is the only control available at present for damping-off and root rot (Kataria and Verma, 1992).

1.3.3 Alternaria brassicae

Alternaria brassicae (Berk.) Sacc. is an economically important pathogen of many cruciferous plants and commonly causes blackspot disease (Verma and Saharan, 1994).
Blackspot is a major disease of rapeseed (*B. rapa* and *B. napus*) in Western Canada and around the world (Bains and Tewari, 1987).

*Alternaria brassicae* infects all aerial plant parts, reducing the photosynthetic area and accelerating senescence and defoliation (Bains and Tewari, 1987). Leaf spotting is the major symptom associated with *Alternaria* infection (Howard et al., 1994). Pre- and post-emergence dumping-off and damage to the inflorescence of seed crops and to seed can also occur. Pinpoint spots on leaves enlarge to become circular lesions several centimeters in diameter with target-like concentric rings. Lesions are initially yellow-brown and later turn brown to black. Fruit infections result in premature shattering leading to seed losses during harvesting (Bains and Tewari, 1987). Precise yield loss estimates have not been made but in artificially inoculated field crops, yield losses of 63% and 42% have been reported for *B. rapa* cv. Span and *B. napus* cv. Zephyr, respectively. The oil and protein content of the seed are also significantly reduced, particularly in *B. rapa*.

The fungus produces large numbers of spores which may spread throughout fields by wind and splashing rain or on equipment, humans and livestock (Howard et al., 1994). Dissemination also occurs as wind-blown diseased plant tissue, although the chief means of spread to new fields is through use of infested seed. *A. brassicae* apparently also survives in susceptible weeds and perennial crops.

In view of the importance of *A. brassicae*, efforts have been made to elucidate the molecular basis of the host-pathogen interaction (Buchwaldt and Green, 1992). In 1987 a phytotoxin, identified as destruxin B (9) (Figure 1.3), was purified from culture filtrates of *A. brassicae* (Ayer and Pena-Rodriguez, 1987; Bains and Tewari, 1987). Bains and Tewari (1987) showed that destruxin B caused damage only on *Brassica* species, leading the authors to consider this toxin to be host-specific. Ayer and Pena-Rodriguez (1987) isolated and identified two more destruxins from *A. brassicae*, homodestruxin B (10) and desmethyl destruxin B (11) (Figure 1.3), but their phytotoxicity was not examined due to limited amounts available (Buchwaldt and Green, 1992).
Figure 1.3 Structures of destruxin B (9), homodestuxin B (10), and desmethyl destruxin B (11)

Chemically, the destruxins are cyclodepsipeptides. Buchwaldt and Green (1992) further examined the phytotoxicity of destruxin B and its possible role in the pathogenesis of *A. brassicae*. Destruxin B, the major phytotoxin produced by *A. brassicae*, was not host-specific on 30 different plant species, causing necrotic and chlorotic symptoms both on host and non-host plants. There were significant differences between taxonomic plant groups in their sensitivity to destruxin B. *Brassica* species were most sensitive to the
toxin, and sensitivity decreased as relatedness of plant groups became more distant: thus it was suggested that destruxin B is host selective in nature. Destruxin B appears to be a virulence factor, contributing to the aggressiveness of *A. brassicae* by conditioning the host tissue and thereby determining the susceptibility of the host (Buchwaldt and Green, 1992).

*Alternaria brassicae* is controlled by hot-water treatment of seeds, which reduces or eliminates both internal and external infestation of seed (Howard et al., 1994). Protective fungicide seed treatments control fungal spores on the seed surface. Long rotations with crops not related to the crucifers and eradication of cruciferous weeds are also helpful control measures.

### 1.4 Phytoalexins

Plants have various defense mechanisms to counter pathogen attack. These mechanisms are both constitutive and induced (Osbourne, 1996; Staskawicz et al., 1995). Among the important induced mechanisms is the production of chemical substances called phytoalexins (Bailey and Mansfield, 1982). These are low molecular weight antimicrobial secondary metabolites produced by plants after exposure to biological, chemical or physical stress. Accumulation of phytoalexins around infection sites is considered an important defense response in plants. Production of phytoalexins by plants after their exposure to microorganisms has been described from over 30 plant families (Brooks and Watson, 1985, 1991; Bailey and Mansfield, 1982). These compounds have been shown to play an important role in plant disease resistance. For example, the phytoalexin resveratrol (12) (Figure 1.4) is responsible for the resistance of grapevine plants to fungal diseases (Hain et al., 1993). Several plants, including grapevine, synthesize the stilbene-type phytoalexin resveratrol when attacked by pathogens. Stilbene biosynthesis requires the presence of the enzyme stilbene synthase. To investigate the potential of stilbene biosynthetic genes in a strategy of engineering pathogen resistance, stilbene synthase
genes were isolated from grapevine, where they are expressed at a high level and transferred into tobacco (Hain et al., 1993). Regenerated tobacco plants containing these genes were found to be more resistant to infection by the fungal pathogen Botrytis cinerea. This was an example of increased disease resistance in transgenic plants based on an additional foreign phytoalexin.

![Chemical Structure](image)

**Figure 1.4** The phytoalexin resveratrol (12).

Phytoalexin biotransformation and detoxification is important for pathogens to overcome plant defenses (VanEtten et al., 1989, 1995). For example, Nectria haematococca, a pea (Pisum sativum) fungal pathogen uses detoxification to degrade the pea phytoalexin pisatin (13) to the less toxic compound 14 (Scheme 1) (Schäfer et al., 1989). The detoxifying activity is due to a substrate-inducible cytochrome P-450 monooxygenase pisatin demethylase (pda). When a cloned gene (PDA-T9) encoding pda was transformed into a nonpathogenic pda− strain of N. haematococca, the recombinant strain became pathogenic (Schafer et al., 1989). These results indicated that pda is required by N. haematococca for pathogenicity on pea and that pisatin itself is a plant defense factor. Among the crucifers, several examples have shown that fungal pathogens are capable of transforming and detoxifying cruciferous phytoalexins. For example, brassinin (1), one of the first phytoalexins reported from crucifers was metabolized to less
toxic products by the blackleg fungus (Pedras et al., 1992). These metabolic transformations of brassinin are discussed in section 1.4.1.3.

**Scheme 1**

1.4.1 Phytoalexins from Crucifers

Phytoalexins from crucifers were first reported by Takasugi (1986) and co-workers. Since then numerous phytoalexins from crucifers have been isolated and characterized. Phytoalexins from the crucifers are the subject of recent reviews (Pedras et al., 1997; Gross, 1993). Since the last review two additional indole phytoalexins have been reported. These are sinalexin (15) from *Sinapis alba* (Pedras and Smith, 1997) and N-methylcamalexin (16) from *Capsella bursa-pastoris* (shepherd’s purse) (Jimenez et al., 1997). The structures of these two phytoalexins are shown in Figure 1.5. Cruciferous phytoalexins possess an indole or indole-related ring system with one or two sulfur atoms as a common feature. These compounds are unique in the sense that they are the only group of phytoalexins known to contain sulfur. As observed with phytoalexins from other plant families, cruciferous phytoalexins are biologically active and possess antifungal and antibacterial properties. Brassinin (1), one of the first phytoalexins reported from *Brassica* species contains a dithiocarbamate group attached to a 3-methylindolyl moiety (Takasugi et al., 1986). Although dithiocarbamates have long been known as important pesticides and
herbicides, so far crucifers appear to be the only plants producing these compounds (Pedras and Taylor, 1993). A number of phytoalexins from crucifers have been shown to inhibit the growth of cultures of human cancer cells (Mehta et al., 1995). As such they could find use in cancer chemotherapy.

![Chemical structures](image)

**Figure 1.5** Structures of sinalexin (15) and N-methylcamalexin (16).

The biosynthesis of cruciferous phytoalexins is still the subject of investigation. L-tryptophan (17) (Figure 1.6) is the biogenetic precursor of some of the indole phytoalexins from crucifers. Recent reports have shown that anthranilate (18) but not tryptophan is a precursor of the phytoalexin camalexin (38) (Tsuji et al., 1993). The biosynthesis of cruciferous phytoalexins is discussed in section 1.4.1.2.

![Chemical structures](image)

**Figure 1.6** Structures of L-tryptophan (17) and anthranilate (18).
1.4.1.1 Synthesis

Relatively large amounts of phytoalexins are required to study their in planta biosynthesis, biotransformation by phytopathogenic fungi and biological activity. Sufficient quantities for such studies are obtainable through synthesis as isolation from plants does not afford sufficient quantities. Twenty-four phytoalexins have been reported from cruciferous plants and synthetic methods are available for fifteen of them.

Brassinin (1) was synthesized from indole-3-carboxaldehyde (19) in a three step procedure (Scheme 2) (Takasugi et al., 1988).

Scheme 2

\[ \text{CHO} \quad \rightarrow \quad \text{N-OH} \]

\[ 19 \rightarrow 20 \]

\[ \text{NH-SCH}_3 \quad \rightarrow \quad \text{NH}_2 \]

\[ 1 \rightarrow 21 \]

\[ ^{a} \text{Reagents: (a) NH}_2\text{OH.HCl; (b) Devarda's alloy, NaOH} / \text{MeOH; (c) Et}_3\text{N, py, CS}_2; \text{MeI}. \]
The aldehyde was treated with hydroxylamine hydrochloride to give a mixture of (E)- and (Z)-oximes (20). The oxime mixture was reduced with Devarda's alloy to yield indole-3-methanamine (21). Treatment of the amine with carbon disulfide in the presence of pyridine and triethylamine gave a dithiocarbamate salt, which was subsequently methylated with methyl iodide to give brassinin (1). A modification of this procedure employing shorter reaction time and resulting in a higher yield of brassinin was also reported (Pedras et al., 1992). By using $^{2}$H$_3$I in the final step, deuterated brassinin was obtained for use in biosynthetic studies (Monde et al., 1994a). In another three step procedure brassinin (1) was synthesized from indole (22) in 58% overall yield (Yamada et al., 1993). In this process (Scheme 3) indole was converted to gramine (23) and the latter methylated with CH$_3$I to the quaternary salt.

Scheme 3 * 

\[ \text{22} \xrightarrow{a} \text{23} \]
\[ \text{1} \xrightarrow{b} \text{21} \]

* Reagents: (a) HCHO, Me$_2$NH, AcOH; (b) MeI; NH$_2$OH; (c) Et$_3$N, py, CS$_2$; MeI.
Nucleophilic displacement with concentrated ammonia solution yielded indole-3-methanamine (21). Subsequent reaction with CS₂ and CH₃I yielded brassinin (1). Brassinin was also synthesized by employing isothiocyanate 26 as a biomimetic intermediate (Scheme 4) (Kutschy et al., 1998).

Scheme 4

\[ \text{CHO} \quad \text{a, b} \quad \text{N-\text{O-H}} \]

\[ \text{19} \quad \text{19} \quad \text{24} \]

\[ \text{N=\text{C=S}} \quad \text{c} \quad \text{NH₂} \]

\[ \text{26} \quad \text{d} \quad \text{25} \]

\[ \text{NH-\text{SCH₃}} \quad \text{e} \quad \text{f} \quad \text{1} \]

\[ \text{27} \quad \text{1} \]

\(^a\) Reagents: (a) (t-Boc)₂O, 30% NaOH / benzene, n-Bu₄NBr; (b) NH₂OH.HCl; (c) NiCl₂.6H₂O, NaBH₄, MeOH; (d) CS₂, CaCO₃; (e) MeSNa, MeOH; (f) MeSNa, 15-crown-5-ether, piperidine.
In this synthesis indole-3-carboxaldehyde (19) was treated with (t-Boc)$_2$O in sodium hydroxide / benzene and tetrabutylammonium bromide as a catalyst to yield the N-protected aldehyde which reacted with hydroxylamine hydrochloride under standard conditions to provide the oxime as (E)- and (Z)-isomers (24) in high yield. Nickel boride catalyzed reduction with sodium borohydride provided 1-t-Boc-3-aminomethylindole (25). Treatment of the amine (25) with thiophosgene in CH$_2$Cl$_2$ / H$_2$O in the presence of CaCO$_3$ gave isothiocyanate 26. Nucleophilic addition of CH$_3$SNa in MeOH afforded 97% yield of protected brassinin (27). The 1-substituted brassinin derivative 27 was also obtained by treatment of the amine (25) with CS$_2$ and Et$_3$N followed by methylation with CH$_3$I. Deprotection with CH$_3$SNa in the presence of 15-crown-5-ether and piperidine gave brassinin (1) in 92% yield.

Cyclobrassinin (2) was synthesized by cyclization of brassinin (1). Bromination of brassinin (1) with pyridinium bromide perbromide in CH$_2$Cl$_2$ followed by dehydrobromination with DBU and subsequent column chromatography provided cyclobrassinin (2) in 35% yield (Scheme 5) (Takasugi et al., 1988).

**Scheme 5**

![Scheme diagram](image)

Reagents: (a) pyridinium bromide perbromide, CH$_2$Cl$_2$, DBU
The cyclization has also been accomplished with NBS and Et$_3$N in place of pyridinium bromide perbromide and DBU (Mehta et al., 1995). [Methyl-$^{2}$H$_3$]cyclobassinin for use in biosynthetic studies was obtained in 25% yield from [methyl-$^{2}$H$_3$]brassinin by cyclization with pyridinium bromide perbromide and DBU (Monde et al., 1994a). In another procedure (Kutschy et al., 1998) cyclobassinin (2) was synthesized from $\text{N}_2$-protected brassinin (27) prepared from isothiocyanate 26.

Brassilexin (29) was synthesized in 18% overall yield in a three step procedure starting with indole-3-carboxaldehyde (19) (Scheme 6) (Devys and Barbier, 1990a).

Scheme 6

\begin{center}
\begin{align*}
\text{19} & \xrightarrow{a} \text{20} \\
\text{20} & \xrightarrow{b} \text{28} \\
\text{29} & \xrightarrow{c} \text{28}
\end{align*}
\end{center}

\textit{a} Reagents: (a) NH$_2$OH.HCl, EtOH; (b) SCl$_2$ / AcOH; (c) PPA.
The aldehyde was treated with hydroxylamine hydrochloride to yield a mixture of (E)- and (Z)-oximes (20) in 93% yield. Reaction of the oxime mixture with sulfur chloride in acetic acid provided the monosulfide (28) in 67% yield. The monosulfide cyclized to brassilexin (29) at room temperature upon treatment with polyphosphoric acid. In a one-pot procedure also starting with indole-3-carboxaldehyde, brassilexin was synthesized in 30% yield (Devys and Barbier, 1993). The aldehyde was first treated with sulfur monochloride in acetic acid to yield a disulfide. Removal of excess acid followed by treatment with ammonia in absolute MeOH provided brassilexin after column chromatography. In another procedure (Scheme 7) brassilexin (29) was synthesized by oxidative ring contraction of the phytoalexin cyclobrassinin (2) (Devys and Barbier, 1992).

Scheme 7

\[ \begin{align*}
\text{2} & \xrightarrow{a} \text{30} \\
\text{29} & \xrightarrow{b} 
\end{align*} \]

*a Reagents: (a) m-CPBA (b) NaIO₄, MeOH / H₂O.
The latter compound was first oxidized with \textit{m}-chloroperoxybenzoic acid to give the phytoalexin cyclobrassinin sulfoxide (30) in 80\% yield. The sulfoxide was then oxidized with NaIO\textsubscript{4} in MeOH / H\textsubscript{2}O to provide brassilexin (29) in 60\% yield. Direct oxidation of cyclobrassinin with NaIO\textsubscript{4} in MeOH / H\textsubscript{2}O also gave brassilexin in a lower yield (30\%) (Devys and Barbier, 1990b). In a four step procedure brassilexin (29) was also synthesized from 2-indolinethione (31) in 64\% overall yield (Scheme 8) (Pedras and Okanga, 1998a).

\textbf{Scheme 8} \textsuperscript{a}

\begin{align*}
\text{31} & \xrightarrow{a} \text{32} \\
\text{34} & \xrightarrow{b} \text{33} \\
\text{34} & \xrightarrow{c} \text{32} \\
\text{29} & \xrightarrow{d} \\
\end{align*}

\textsuperscript{a} Reagents: (a) NaH, HCOOEt; (b) NH\textsubscript{2}OH.HCl, NaOAc, EtOH; (c) NaBH\textsubscript{3}CN, TiCl\textsubscript{3}, MeOH; (d) Activated carbon.
Formylation of 2-indolinethione (31) with ethyl formate gave 2-mercaptoindole-3-carboxaldehyde (32) in 92% yield. Treatment of this aldehyde with hydroxylamine hydrochloride under standard conditions quantitatively yielded a mixture of (E)- and (Z)-oximes (33). Reduction of the oxime with NaBH₃CN in the presence of TiCl₃ yielded 3-methylenaminoindoled-2-thione (34) (85%). The latter compound was treated with activated charcoal in MeOH to afford brassilexin (29) in 85% yield.

(±)-Dioxibrassinin (36) was synthesized from 3-(aminomethyl)-3-hydroxyindole.HCl (35) by treatment with CS₂ in the presence of triethylamine and pyridine followed by methylation with CH₃I (Scheme 9) (Monde et al., 1991a). This process gave dioxibrassinin (36) in 75% yield. By employing C₂H₃I for the methylation step (±)-[methyl-²H₃]dioxibrassinin was obtained for use in biosynthetic studies (Monde et al., 1994a).

**Scheme 9**

![Scheme 9 Diagram](image)

*a Reagents: (a) CS₂, Et₃N, py; MeI.

Camalexin (38) was synthesized from indole (20) in a two step procedure (Scheme10) (Ayer et al., 1992). The reaction of two equivalents of indolylmagnesium iodide (prepared in situ from indole and methylmagnesium iodide) with 2-bromothiazole
in refluxing benzene afforded camalexin (38) in 68-76% yield. In a four step procedure camalexin was synthesized from 2-trimethylsilyl thiazole (39) (Scheme 11) (Fürstner and Ernst, 1995). The latter compound provided ketone 40 in good yield upon acylation with 2-nitrobenzoyl chloride in CH₂Cl₂. Standard hydrogenation of 40 over Pd on charcoal followed by formylation of the resulting amino group with HCOOH / Ac₂O gave o xoamide 43, which was reductively cyclized. Thus, heating of a suspension of 43, TiCl₃ and zinc dust in DME followed by an extractive work-up afforded camalexin (38) in 71% yield. Camalexin (38) was also synthesized by an alternative procedure (Fürstner and Ernst, 1995) amenable to large scale preparations from 2-bromothiazole (41).

Scheme 10

\[ \text{22} \xrightarrow{a} \text{37} \xleftarrow{b} \text{38} \]

Reagents: (a) MeMgl, Et₂O; (b) 2-bromothiazole, benzene.
Lithiation of 2-bromothiazole (41) and subsequent reaction with 2-nitrobenzaldehyde gave the alcohol (42) in 80% yield, which was oxidized to 40 using PDC in CH₂Cl₂.

**Scheme 11**

![Reaction Scheme]

---

Reagents and conditions: (a) 2-nitrobenzoyl chloride, CH₂Cl₂; (b) n-BuLi, Et₂O, -78 °C; 2-nitrobenzaldehyde, Et₂O, -78 °C; (c) PDC, CH₂Cl₂; (d) H₂, Pd-charcoal, EtOAc; (e) HCOOH, Ac₂O; (f) TiCl₃, Zn-dust, DME, reflux; EDTA disodium salt, H₂O.

6-Methoxycamalexin (49) was prepared from 4-methyl-3-nitroaniline (44) in five steps (Scheme 12) (Ayer et al., 1992). 4-Methyl-3-nitrophenol (45) was prepared in 75% overall yield from 4-methyl-3-nitroaniline (44), and then methylated to yield 4-methyl-3-
nitroanisole (46). Treatment of 4-methyl-3-nitroanisole (46) with dimethylformamide dimethyl acetal and pyrrolidine gave β-pyrrolidinostyrene (47) which was reduced with Ti(III) chloride in ammonium acetate buffer to afford 6-methoxyindole (48) in 73-78% overall yield.

Scheme 12

\[ \text{Reagents: (a) NaN_3, H}_2\text{SO}_4, \text{ H}_2\text{O; (b) Me}_2\text{SO}_4, \text{ K}_2\text{CO}_3, \text{ MeCN; (c) dimethylformamidedimethyl acetal, pyrrolidine; (d) TiCl}_3, \text{ NH}_4\text{OAc; (e) MeMgI, Et}_2\text{O; (f) 2-bromothiazole, benzene.} \]

Alkylation of 6-methoxyindolyl magnesium iodide with 2 equivalents of 2-bromothiazole, according to the procedure used to prepare camalexin, gave 77% yield of 6-methoxycamalexin (49).
Brassicanal A (52) was synthesized by Vilsmeier formylation of 2-(methylthio) indole (51) (Monde et al., 1990). As shown in Scheme 13, thiation of oxindole (50) with phosphorous pentasulfide in refluxing benzene provided 2-indoline thione (31) which on treatment with methyl iodide, Na₂CO₃ in acetone gave 2-(methylthio)indole (51).

Scheme 13

\[ \text{50} \xrightarrow{\text{a}} \text{31} \xrightarrow{\text{d}} \text{32} \]
\[ \text{52} \xleftarrow{\text{c}} \text{51} \xrightarrow{\text{e}} \]

\( ^{a} \text{Reagents and conditions: (a) P₂S₅, benzene, reflux; (b) MeI, CaCO₃, Me₂CO; (c) POCl₃, DMF; (d) NaH, HCOOEt; (e) CH₃N₂, Et₂O.} \)

Vilsmeier formylation of the latter compound provided brassicanal A (52). In another synthesis, brassicanal A (52) was obtained by methylation of 2-mercaptoindole-3-carboxaldehyde (32) with diazomethane (Scheme 13) (Pedras and Okanga, 1998a).

Brassicanal B (53) was synthesized by treatment of 2-indolinethione (31) with bromoacetone followed by Vilsmeier formylation (Monde et al., 1990). This process gave the phytoalexin in 33% yield (Scheme 14).
Scheme 14

![Chemical structures](image)

R{eq}^a\text{Reagents: (a) bromoacetone; (b) POCl}_3, \text{DMF.}

1-Methoxybrassinin (58) was synthesized in seven steps from indole (22) with an overall yield of 22% (Scheme 15) (Yamada et al., 1993; Somei et al., 1992). Thus, indole (22) was converted to gramine (23) which yielded indole-3-methanamine (21) upon treatment with CH$_3$I and concentrated ammonia solution. Indole-3-methanamine (21) was then converted to its trifluoroacetyl derivative (54) by treatment with ethyl trifluoroacetate in THF. Reduction with triethylsilane in trifluoroacetic acid afforded 2,3-dihydroindole (55). Catalytic oxidation of 55 with Na$_2$WO$_4$·2H$_2$O and 30% H$_2$O$_2$, followed by methylation with CH$_2$N$_2$ produced 56 in 77% yield. Subsequent alkaline hydrolysis in methanol-water produced 3-aminomethyl-1-methoxyindole (57) in 98% yield. The latter compound was readily converted to methoxybrassinin (58) by treatment with CS$_2$ and MeI in 64% yield.
Starting from isothiocyanate 26, brassitin (61) was synthesized in three steps (Scheme 16) (Kutschy et al., 1998). Treatment of 26 with 32 equivalents of CH₃ONa in MeOH gave compound 59 which was methylated to yield 60. The latter compound was
selectively hydrolyzed under acidic conditions to afford brassitin (61) in 39% yield. Brassitin (61) was also prepared by oxidation of brassinin with 30% aq H₂O₂ in the presence of p-toluenesulfonic acid and triphenylphosphine (Monde et al., 1995a). This afforded the phytoalexin in 8% yield.

Scheme 16

\[ \begin{align*}
&\text{N= CSCl} \\
&\Downarrow \text{a} \\
&\text{H} \\
&\text{26} \\
&\text{N= OSO}_{2}\text{Me} \\
&\Downarrow \text{b} \\
&\text{H} \\
&\text{59} \\
&\text{N= SO}_{2}\text{Me} \\
&\Downarrow \text{c} \\
&\text{H} \\
&\text{60} \\
&\text{N= OCH}_{3} \\
&\Downarrow \text{61} \\
&\text{N= OCH}_{3} \\
&\text{H} \\
\end{align*} \]

\( a \) Reagents: (a) MeONa, MeOH; (b) MeONa, MeOH; MeI; (c) HCl (1:1), THF.

4-Methoxybrassinin (65) was synthesized from indole-3-carboxaldehyde in 4 steps (Scheme 17) (Yamada et al., 1993). Thus, indole-3-carboxaldehyde (19) was converted to 4-methoxyindole-3-carboxaldehyde (62) followed by reaction with NaBH₄ in Me₂NH to give 3-dimethylaminomethyl-4-methoxyindole (63). Subsequent reaction with NaBH₄
in NH₄OH produced 4-methoxyindole-3-methanamine (64) in 74% yield. Treatment with CS₂ and MeI afforded 4-methoxybrassinin (65) in 64% yield.

Scheme 17

\[ \text{Scheme 17} \]

![Scheme diagram](image)

\[ \text{19} \xrightarrow{a} \text{62} \xrightarrow{b} \text{63} \]

\[ \text{65} \xrightarrow{d} \text{64} \]

\[ \text{a Reagents: (a) Ti(CF₃COO)₃, CF₃COOH; I₂, CuI, DMF; NaOMe; (b) NaBH₄, Me₂NH; (c) NaBH₄, NH₄OH; (d) CS₂, Et₃N, py; MeI.} \]

Dehydro-4-methoxycyclobassinin (67) was obtained in two steps from 4-methoxybrassinin (Scheme 18) (Monde et al., 1994b). Treatment of 4-methoxybrassinin (65) in CH₂Cl₂ with NBS followed by dehydrobromination with DBU afforded 4-methoxycyclobassinin (66) in 20% yield. Refluxing of the benzene solution of 66 with DDQ gave dehydro-4-methoxycyclobassinin (67) in 34% yield.
Scheme 18 a

![Chemical structure](image)

\(\text{65} \xrightarrow{a} \text{66} \xrightarrow{b} \text{67}\)

\(^a\) Reagents and conditions: (a) NBS, \(\text{CH}_2\text{Cl}_2\); DBU; (b) DDQ, benzene, reflux.

\((\pm)-3\)-Cyanomethyl-3-hydroxyxindole (69) was synthesized in 48% yield (Scheme 19) (Monde et al., 1991a) by treatment of acetonitrile with \(n\)-BuLi in THF followed by addition of isatin (68).

Scheme 19 a

![Chemical structure](image)

\(\text{68} \xrightarrow{a} \text{69}\)

\(^a\) Reagents: (a) MeCN, \(n\)-BuLi, THF.

31
Methoxybrassinenin A (70) was obtained by methylation of methoxybrassinin (58) with MeI and K$_2$CO$_3$ (Scheme 20) (Monde et al., 1991b).

Spirobrassinin (71) was synthesized by treatment of dioxibrassinin with thionyl chloride (Monde et al., 1994a). The structure of spirobrassinin is shown in Figure 1.7.

Scheme 20

\[ \text{58} \xrightarrow{\text{a}} \text{70} \]

*Reagents: (a) K$_2$CO$_3$, MeI, MeOH.*

No syntheses have been reported for the cruciferous phytoalexins sinalexin (15), N-methylcamalexin (16) (Figure 1.5) brassicanal C (72), N-methoxyspirobrassinol (73), N-methoxyspirobrassinol methyl ether (74), cyclobrassinone (75), 1-methoxyspirobrassinin (76), methoxybrassitin (77) and methoxybrassenin E (78) (Figure 1.7).
Figure 1.7 Structures of the phytoalexins spirobrassinin (71), brassicanal C (72), N-methoxyspirobrassinol (73), N-methoxyspirobrassinol methyl ether (74), cyclobassinone (75), methoxyspirobrassinin (76), methoxybrassitin (77) and methoxybrassinen B (78).

1.4.1.2 Biosynthesis

The amino acid L-tryptophan (17) is the precursor of indole alkaloids including some of the cruciferous phytoalexins (Monde et al., 1994a). Tryptophan is biosynthesized via the shikimate pathway. The biosynthesis of shikimate metabolites is the subject of several reviews, one of which is very recent (Dewick, 1998). As shown in Scheme 21, the first reaction in the shikimate pathway involves the aldol-like condensation of phosphoenolpyruvate (PEP) (79) with D-erythro 4-phosphate (80) giving 3-deoxy-D-
Scheme 21

Enzymes: (a) DAHP synthase; (b) 3-dehydroquinate synthase; (c) 3-dehydroquinase; (d) shikimate dehydrogenase; (e) quinate dehydrogenase.
arabino-heptulosonate-7-phosphate (DAHP) (81), a reaction catalyzed by the enzyme DAHP synthase (phospho-2-dehydro-deoxyheptonate aldolase). 3-Dehydroquinate synthase catalyses the conversion of DAHP (81) into 3-dehydroquinate (82) by a sequence of reactions requiring an oxidation, a β-elimination, a reduction and an intramolecular aldol condensation. The dehydration of 3-dehydroquinate (82) to 3-dehydroshikimate (83) (Scheme 21) is catalyzed by 3-dehydroquinase (3-dehydroquinate dehydratase). Reduction of (83) then provides shikimate (84). Similarly, reduction of (82) gives quinate (85). Phosphorylation of shikimate (84) to shikimate 3-phosphate (86) is brought about by shikimate kinase in the presence of ATP (Scheme 22). EPSP synthase (3-phosphoshikimate 1-carboxyvinyltransferase) catalyzes the condensation of shikimate 3-phosphate (86) with PEP to produce the enol ether 5-enolpyruvylshikimate 3-phosphate (EPSP) (87) (Scheme 22). The elimination of phosphoric acid from EPSP (87) yields chorismate (88) and is catalyzed by the enzyme chorismate synthase (5-enolpyruvylshikimate 3-phosphate-lyase). L-Tryptophan (17) is formed from chorismate (88) via anthranilate (18) as shown in Scheme 23. A feature of this pathway is the Amadori rearrangement on phosphoribosylantranilate (89) catalyzed by the enzyme phosphoribosylantranilate isomerase. The keto amine (90) is then converted to indole-3-glycerol phosphate (91) through catalysis by indole-3-glycerol synthase. Tryptophan synthase catalyses the final reaction in the sequence, transformation of indole-3-glycerol phosphate (91) plus L-serine into L-tryptophan (17).
Scheme 22

$\text{Scheme 22}^a$

\[ \text{84} \xrightarrow{\text{a, ATP}} \text{86} \]

\[ \text{88} \xrightarrow{\text{b, PEP}} \text{87} \]

$^a$ Enzymes: (a) shikimate kinase; (b) EPSP synthase; (c) chorismate synthase.

It is a multienzyme complex (the $\alpha_2\beta_2$ complex), comprising two $\alpha$ subunits and a $\beta_2$ dimeric subunit. The $\alpha$ subunits also catalyze aldolytic cleavage of indole-3-glycerol phosphate, and, by use of the cofactor PLP, the $\beta$ subunits catalyze reaction of L-serine and indole (22), giving L-tryptophan. The overall reaction is catalyzed only by the $\alpha_2\beta_2$ complex, and indole (22) is not normally released as an intermediate, but is channelled between the active sites via a hydrophobic tunnel.
Scheme 23

a Enzymes: (a) anthranilate synthase (TrpE, TrpG); (b) anthranilatephosphoribosyltransferase (TrpD); (c) phosphoribosylantranilate isomerase (TrpF); (d) indole 3-glycerol phosphate synthase (TrpC); (e) tryptophan synthase (TrpA, TrpB); a and b refer to subunits of tryptophan synthase.
The biosynthesis of the phytoalexins brassinin (1), cyclobassinin (2) and spirobrassinin (71) was studied (Monde and Takasugi, 1991; Monde et al., 1994a) using labelled precursors administered to turnip tissue obtained from turnip root (*B. campestris* L. ssp. *rapa*). The biosynthetic pathway that was proposed is shown in Scheme 24. In order to confirm the origin of an indole moiety of brassinin, incorporation of L-tryptophan was examined. When L-[4′-2H]tryptophan was fed to UV-irradiated turnip tissue and incubated for 37 hours, spirobrassinin (71) was isolated as the main metabolite. Spectroscopic analysis revealed incorporation of the 2H label into the oxindole nucleus, of 71. A feeding experiment with L-[methyl-2H₃]methionine indicated that the methyl groups of brassinin, cyclobassinin and spirobrassinin arise from L-methionine. Administration of [methyl-2H₃]brassinin to the turnip tissue, followed by 27 hours incubation led to effective incorporation of the 2H label into cyclobassinin (2) and spirobrassinin (71). These results indicate that the biological origin of these phytoalexins is L-tryptophan and that brassinin (1) is an advanced precursor of cyclobassinin (2) and spirobrassinin (71). However, cyclobassinin (2) and dioxibrassinin (36) (Monde et al., 1994a), a plausible precursor to spirobrassinin (71), were not incorporated into spirobrassinin (71).

Time-course studies of phytoalexins and glucosinolates in UV-irradiated turnip root tissue (*B. campestris* L. ssp. *rapa*) indicated that both the levels of phytoalexins and indole glucosinolates increased in the UV-irradiated tissue whereas only the latter did in non-irradiated control tissue (Monde et al., 1991). Glucosinolates, which are abundant among cruciferae, give their respective isothiocyanates on enzymic hydrolysis by the co-existing enzyme myrosinase, followed by Lossen-type rearrangement. Brassinin (1) shows a striking structural similarity to indol-3-ylmethyl isothiocyanate (93), the supposed enzymic hydrolysis product of indole glucosinolate, glucobrassicin (92) (Scheme 24). These facts suggested the possibility that some biosynthetic relationships may exist between the indolic phytoalexins and indole glucosinolates (Monde et al., 1994a).
Scheme 24

\[
\text{L-cysteine} \quad \text{92} \quad \text{Myrosinase} \quad \text{93}
\]

\[
\text{L-methionine} \quad \text{1}
\]

\[
\text{Other indole phytoalexins}
\]

\[
\text{71} \quad \text{17} \quad \text{2}
\]
It is known that glucosinolates are biosynthesized through a common biosynthetic pathway from an amino acid precursor. In the case of indole glucosinolate, glucobrassicin (92), it is biosynthesized from L-tryptophan (17) (Scheme 24). Therefore, brassinin (1) would be derived from L-tryptophan (17), though it is not clear whether the indole or indole-related phytoalexins are biosynthesized from indole glucosinolates or directly from tryptophan (Monde et al., 1994a).

A crucial point in the biosynthetic pathway to brassinin is whether the thiocarbonyl carbon of brassinin, therefore, the relevant imino carbon in spirobrassinin, originates from the C-2 carbon of tryptophan (Monde and Takasugi, 1991). If that is the case, then the biosynthetic pathway to brassinin should involve a molecular rearrangement step since the thiocarbonyl carbon of brassinin is separated from the methylene carbon by a nitrogen atom. A feeding experiment with DL-[2-13C]tryptophan resulted in a four-fold enhancement of the imino carbon NMR signal of spirobrassinin and indicated the involvement of a molecular rearrangement in the pathway from tryptophan to brassinin (Monde and Takasugi, 1991). This result was suggestive of the isothiocyanate (93) as a key intermediate to brassinin. To examine the possible role of isothiocyanates in the biosynthesis of cruciferous phytoalexins, benzyl isothiocyanate was chosen as a model substrate and administered to the turnip tissue (Monde and Takasugi, 1991). A new metabolite was isolated and identified as PhCH₂NH-CS-SMe by direct comparison with a synthetic specimen. Formation of this metabolite in the turnip tissue suggested strongly that indol-3-ylmethyl isothiocyanate (93) (Scheme 24) would be involved in the biosynthesis of brassinin. It remains to be proved whether isothiocyanate 93 could be formed via hydrolysis of the indole glucosinolate (92) and / or directly from L-tryptophan (17) e.g. via thiohydroxamic acid. In order to detect the labile indol-3-ylmethyl isothiocyanate intermediate, a trapping experiment with methanethiolate was done (Monde et al., 1994a). UV-irradiated turnip roots were homogenized with 15% aqueous sodium methanethiolate and the homogenate extracted with ethyl acetate. Separation of the extract
gave two adducts. Spectroscopic data and direct comparison with the authentic samples revealed that they were brassinin and PhCH₂-NH-CS-SCH₃, which was named phenylbrassinin. Neither brassinin nor phenylbrassinin was isolated in the absence of CH₃SNa. The isolation of brassinin suggested a transient formation of isothiocyanate 93 as a reaction intermediate.

Scheme 25

There are two possible biosynthetic routes from unstable isothiocyanate 93 to brassinin (Scheme 25). Path A involves a direct attack by a methylthio group from L-methionine whereas path B is a stepwise mechanism via dithiocarbamic acid intermediate 94, or its equivalent (Monde et al., 1994a). In order to clarify whether the methylthio
group in the phytoalexin is introduced directly or in a stepwise manner, a mixture of L-[methyl-\textsuperscript{3}H\textsubscript{3}] methionine and L-[\textsuperscript{35}S]methionine was fed to the UV-elicitated turnip tissue (Monde et al., 1994a). The mixture was considered doubly labelled L-[methyl-\textsuperscript{3}H\textsubscript{3}, \textsuperscript{35}S]methionine. The radioactive ratio of \textsuperscript{35}S / \textsuperscript{3}H in the L-methionine mixture was 2.4. Forty-eight hours after administration of the mixture, metabolites were extracted with ethyl acetate and separated to yield brassinin and sprobrassinin. The ratio of \textsuperscript{35}S / \textsuperscript{3}H of isolated brassinin and sprobrassinin was 2.5 and 2.2 at several concentrations, respectively. Therefore, the ratio was considered unchanged during biosynthesis of brassinin and sprobrassinin. This result indicated unambiguously that brassinin is biosynthesized by intact incorporation of the methylthio group from L-methionine into the isothiocyanate (93). The same results were obtained when several experiments were done using L-methionine with different ratios of \textsuperscript{35}S / \textsuperscript{3}H. Since the two sulfur atoms of glucobrassinin are derived from L-cysteine, the sulfur atom of the isothiocyanate intermediate (93) and the thiocarbonyl sulfur atom of brassinin are most likely derived from L-cysteine (Monde et al., 1994a). To confirm the origin of the other sulfur atom, feeding experiments with L-[\textsuperscript{35}S] cysteine were examined. The radioactivities of isolated brassinin and sprobrassinin were clearly high. These results supported the conclusion that the thiocarbonyl sulfur atom originates from L-cysteine.

Production of a variety of indole or indole-related phytoalexins can be explained by postulating a common intermediate which links brassinin and other cruciferous phytoalexins (Monde et al., 1994a). To determine the nature of the intermediate, 2-methylbrassinin (95) (Scheme 26) was chosen as a probe. Since the compound (95) is blocked by a methyl group at the C-2 position, the formation of a cyclobrassinin-type or a sprobrassinin-type compound is structurally prohibited. Therefore, possible isolation of a corresponding intermediate was expected. Synthetic 95 was administered to UV-elicited turnip tissue. After a 27 hour incubation period, two new compounds were isolated which were not detected in the control tissue.
Spectroscopic analysis led to their identity as 97 and 98. These two metabolites, especially the compound 98, gave important information pertaining to the intermediate in question. Formation of indoxyl 98 (Scheme 26) (Monde et al., 1994a) suggested that the immediate precursor of 98 is diol 96, which gives rise to 98 in a pinacol-type rearrangement. The thiocarbamate side chain of 98 may be derived from the corresponding dithiocarbamate by oxidation. The supposed structure of diol intermediate 96 could account for the formation of metabolite 97. Dehydration of 96, followed by internal displacement could lead to spiro compound 97. Furthermore, the carbonyl group
of thiocarbamate 98 is less nucleophilic as compared with the thiocarbonyl group of 96. Therefore, rearrangement would precede cyclization in diol 96.

The co-occurrence of methoxybrassinin (58) with brassinin (1) and cyclobrassinin (2) in *P. cichorii*-inoculated Chinese cabbage (Takasugi et al., 1986) and Japanese radish suggested the possibility that N-hydroxylation of brassinin, followed by biological methylation gives methoxybrassinin, which leads to cyclobrassinin through eliminative cyclization (Monde et al., 1995b). When [methyl-²H₃] brassinin was fed to Japanese radish inoculated with *P. cichorri* and incubated for 7 hours, cyclobrassinin and methoxybrassinin were isolated (Monde et al., 1995b). Mass spectroscopic analysis showed that the ²H label was incorporated into cyclobrassinin. This result indicated that cyclobrassinin is biosynthesized from brassinin as reported in the case of turnip root (Monde et al., 1994a; Monde and Takasugi, 1991). If cyclobrassinin is biosynthesized through methoxybrassinin, the latter should contain the deuterium label. However, no ²H label was present in isolated methoxybrassinin, indicating the lack of incorporation of brassinin into cyclobrassinin through methoxybrassinin. A feeding experiment with [methyl-²H₃] methoxybrassinin (Monde et al., 1995b) supported this conclusion. Furthermore, mass spectral analysis of the isolated cyclobrassinin showed the absence of a molecular ion peak due to [methyl-²H₃] cyclobrassinin. These results indicated that cyclobrassinin is not biosynthesized from methoxybrassinin, and that methoxybrassinin is not derived from brassinin.

The biosynthesis of indole phytoalexins in kohlrabi (*B. oleracea* var. gongylodes) has also been studied (Gross et al., 1994) using radiolabelled precursors. Feeding both L-[2-¹⁴C]tryptophan and L-[¹⁴CH₃]methionine to UV-irradiated stem tubers of kohlrabi indicated that the S-containing indole phytoalexins were biosynthesized from these amino acids.

It is not clear how phytoalexins that contain only one sulfur atom, such as brassicanal A (Monde et al., 1990) are biosynthesized. This group of phytoalexins is thought to be
biosynthesized from indole phytoalexins that contain two sulfur atoms. In a unique trapping of a potential biosynthetic intermediate to brassicanal A, aniline or acetanilide was added to UV-irradiated turnip tissue (Monde et al., 1996). The structure of the trapping product (101) (Scheme 27) strongly suggested that 2-mercaptoindole-3-carboxaldehyde (32) is a biosynthetic intermediate from brassinin to brassicanal A. The expected precursor (32) or its thione tautomer may be derived by hydrolysis of dehydrocyclobrassinin (99), which in turn would be formed from 2 via enzymatic dehydrogenation (Scheme 27) (Monde et al., 1996). Although dehydrocyclobrassinin has not yet been isolated, a methoxy derivative (67) was isolated from Pseudomonas cichorii-inoculated turnip root (Monde et al., 1994a). Co-occurrence of brassicanal A (52) with cyclobrassinin (2) in P. cichorii-inoculated Chinese cabbage (Monde et al., 1990) supports this pathway.

Brassinin (1) and cyclobrassinin (2) have been shown to be intermediates in the biosynthesis of the cruciferous phytoalexin brassilexin (29) (Pedras et al., 1998). Following feeding experiments with the tetradeuterated cruciferous phytoalexins brassinin and cyclobrassinin, leaves of Brassica carinata were elicited with the blackleg causing fungus P. lingam and incubated. Spectroscopic and HPLC analyses indicated that both brassinin and cyclobrassinin were incorporated into the cruciferous phytoalexin brassilexin.

Anthranilate (18), but not tryptophan (17), was shown to be a biosynthetic precursor of camalexin through the use of tryptophan-deficient mutants of Arabidopsis and feeding the detached leaves of Arabidopsis with radiolabelled anthranilate and tryptophan (Tsuji et al, 1993). It was initially hypothesized that camalexin is biosynthesized from tryptophan because both compounds have an indole moiety. However, the report (Wright et al., 1991) which showed anthranilate and not tryptophan to be a precursor of indole-3-acetic acid, led to speculation that anthranilate rather than tryptophan is a precursor of camalexin (Tsuji et al., 1993). To test this possibility,
Scheme 27

1 \rightarrow 2 \rightarrow 99 \quad (R = H)

67 \quad (R = OCH₃)

52 \rightarrow \begin{bmatrix} 32 \end{bmatrix} \rightarrow 100

Aniline

101
equimolar amounts of radiolabelled tryptophan and anthranilate of the same specific activity were fed to detached leaves of *Arabidopsis* followed by treatment with AgNO₃ (Tsuji et al., 1993). The specific activity of camalexin labelled with [¹⁴C] anthranilate was 5 to 6-fold greater than the specific activity of camalexin labelled with [²⁵H] tryptophan.

As an additional test of whether or not camalexin is synthesized from tryptophan, camalexin levels in three tryptophan-requiring mutants of *Arabidopsis* were measured after treatment with AgNO₃ (Tsuji et al., 1993). The results of these experiments supported the hypothesis that anthranilate but not tryptophan is a direct biosynthetic precursor of camalexin in *Arabidopsis*. Based on these results, it has been hypothesized that the camalexin biosynthetic pathway originates from an intermediate of the tryptophan pathway which lies between anthranilate and indole, most likely indole-3-glycerol phosphate (91) (Tsuji et al., 1993).

Browne et al., (1991) first proposed a possible route of camalexin biosynthesis that involves the condensation of indole-3-carboxaldehyde (19) with cysteine followed by cyclization and decarboxylation. Based on this prediction of the biosynthesis of the thiazole ring of camalexin, Zook and Hammerschmidt (1997) have formulated a detailed hypothetical biosynthetic pathway (Scheme 28). According to this pathway, the formation of the thiazole ring of camalexin (38) would involve two oxidation steps followed by a decarboxylation of the intermediate indole-3-thiazolidinecarboxylic acid (102).

To determine the possible origin of the sulfur atom in the thiazole ring of camalexin, detached noninoculated leaves of *Arabidopsis* and leaves inoculated with the maize pathogen *C. carbonum* were fed [³⁵S]cysteine and [³⁵S]methionine (Zook and Hammerschmidt, 1997). Inoculation of *Arabidopsis* leaves with *C. carbonum* elicited camalexin production. A comparison was made between these two labelled amino acids in terms of the efficiency of the incorporation of radioactivity into camalexin. Labelled serine was also included in this study because serine is a precursor of cysteine via O-acetylserine in plants. There was a large increase in the incorporation of radioactivity from cysteine
into camalexin from inoculated leaves, as compared with control leaves. The magnitude of this difference was much smaller when labelled serine, or methionine was fed to detached leaves. The incorporation of radioactivity into camalexin from $[^{35}\text{S}]$ cysteine was more than 10-fold greater than the incorporation of radioactivity from $[^{35}\text{S}]$ methionine or $[^1\text{H}]$ serine for *Arabidopsis* leaves inoculated with *C. carbonum*. Likewise, the specific activity of labelled camalexin was more than 5-fold greater when $[^{35}\text{S}]$ cysteine was used as the source of radioactivity, as compared with $[^{35}\text{S}]$methionine. These results provided strong evidence that the sulfur atom of the thiazole ring of camalexin originates from cysteine.

Scheme 28
In order to determine whether part of the carbon backbone of cysteine is also incorporated into camalexin, \([^{14}\text{C}]\text{cysteine}\) was fed to noninoculated and inoculated leaves of *Arabidopsis* (Zook and Hammerschmidt, 1997). High levels of incorporation of \([^{35}\text{S}]\text{cysteine}\) and \([^{14}\text{C}]\text{cysteine}\) into camalexin were observed. These results suggested that both the sulfur and carbon atoms from cysteine are incorporated into the thiazole ring of camalexin.

To obtain direct evidence that a portion of the cysteine molecule is incorporated intact into camalexin, cysteine was labelled with stable isotopes for mass spectrometric analysis. Cysteine labelled with either \(^2\text{H}\) or \(^{13}\text{C}\) and \(^{15}\text{N}\) was fed through petioles of *C. carbonum* inoculated leaves of *Arabidopsis* (Zook and Hammerschmidt, 1997). Labelled camalexin was then extracted and analyzed by mass spectrometry. The ratio of intensities of mass ion fragments for the molecular ion (203 / 200) and fragmentation of the thiazole ring (60 / 58 and 143 / 142) were found to be consistent with the formation of the thiazole ring from cysteine.

At present there is no direct evidence for indole-3-carboxaldehyde as a biosynthetic precursor of camalexin in *Arabidopsis*. Indole-3-carboxylic acid has been proposed (Zook and Hammerschmidt, 1997) as an equally plausible biosynthetic precursor of camalexin.

### 1.4.1.3 Microbial biotransformations

The biotransformation and detoxification of phytoalexins by phytopathogenic fungi is an evolutionary process whereby pathogens can circumvent resistance mechanisms of plants. To date several examples demonstrate that fungal pathogens can detoxify cruciferous phytoalexins efficiently (Pedras et al., 1997). Multiple examples of phytoalexin detoxification have been reported in other plant families (Daniel and Purkayastha, 1995; VanEtten et al., 1989).
The biotransformation of the phytoalexin brassinin by the blackleg fungus and related species was investigated (Pedras and Taylor, 1991, 1993; Pedras et al., 1992). It was determined that the virulence of the blackleg fungus correlated with its ability to rapidly metabolize and detoxify brassinin (Pedras et al., 1992). Incubation of the virulent \textit{P. lingam} isolates with brassinin (1) resulted in the detection and isolation of three metabolites. The unusual structure of the first metabolic intermediate was assigned as methyl (3-indolylmethyl) dithiocarbamate S-oxide (103) based on spectroscopic data and synthesis of a methyl derivative. The other two metabolites were readily identified as indole-3-carboxaldehyde (19) and indole-3-carboxylic (104) acid from spectroscopic data and comparison with authentic samples. Further biotransformation studies on the three metabolites by virulent \textit{P. lingam} established the biotransformation pathway from brassinin as shown in Scheme 29.

\textbf{Scheme 29}
Further investigation (Pedras and Taylor, 1993) on the metabolism of brassinin indicated that avirulent isolates of *P. lingam* follow a metabolic pathway which is different from that of virulent isolates. The metabolism of brassinin (1) by avirulent isolates resulted in the detection and isolation of four metabolites. The structures of three metabolites were readily determined to be indole-3-carboxaldehyde (19), Indole-3-carboxylic acid (104) and indole-3-methanamine (21) by comparison of their spectroscopic data with those of authentic samples. The structure of the fourth metabolite was deduced from spectroscopic data to be N-acetyl-3-indolyl-methylanmine (105) and confirmed by synthesis. Each of the four metabolites was subjected to further biotransformation by the avirulent *P. lingam* isolates. From the results of these studies, the metabolism of brassinin by these isolates was established as represented in Scheme 30. The biotransformation occurred in a series of steps in which the initial intermediate was either acetylamine 105 or amine 21 and the last one the acid (104). Thus a striking difference in the metabolism of brassinin by avirulent and virulent isolates occurred. While in virulent isolates the transformation of brassinin rapidly yielded aldehyde 19 via intermediate 103 (Pedras and Taylor, 1991; Pedras et al., 1992), in avirulent isolates brassinin was slowly converted to aldehyde 19 via intermediates 21 and 105. The biotransformation of brassinin was much slower with the avirulent isolate than with the virulent one (more than 5 days for avirulent vs 24 hours for virulent to yield the final product (104).

The antifungal activity of brassinin and its metabolites was compared (Pedras and Taylor, 1993) using spore germination and radial mycelial growth assays. Methyl (3-indolylmethyl) dithiocarbamate S-oxide, and indole-3-carboxaldehyde were less active than brassinin in the spore germination assay. Indole-3-carboxylic acid did not have a detectable effect on either spore germination or radial mycelial growth. These results indicated that the biotransformation of brassinin by *P. lingam* isolates is a detoxification
process. In addition, it was established that brassinin inhibited the biosynthesis of nonselective phytotoxins by \textit{P. lingam}. These phytotoxins were not detected in fungal cultures incubated with brassinin until 6-8 hours after complete brassinin transformation. By contrast, incubation with any of the intermediates 21, 103, 104, 105 did not noticeably affect phytotoxin production.

\textbf{Scheme 30}

\begin{align*}
\text{1} & \quad \text{21 } R = \text{H} \\
\text{104} & \quad \text{105 } R = \text{COCH}_3
\end{align*}

The biotransformation of the cruciferous phytoalexin brassicanal A (52) by the blackleg fungus led to the detection and isolation of three metabolites (Pedras and Khan, 1996). Structure determination by spectroscopic methods and concomitant synthesis led to their identification as brassicanal A sulfoxide (106), 3-hydroxymethylindole 2-
methylsulfoxide (107), and 3-methylindole 2-methylsulfoxide (108). Compared to virulent isolates of the fungus, the metabolism of brassicanal A occurred much more slowly with avirulent isolates. The antifungal activities (to virulent blackleg isolates) of brassicanal A and its biotransformation products were compared using a spore germination assay. The results of these assays indicated that brassicanal A was significantly more inhibitory to blackleg spore germination than its biotransformation products. The biotransformation of brassicanal A by the blackleg fungus is therefore a detoxification process. The sequence of reactions that brassicanal A (52) undergoes when incubated with virulent isolates of *P. lingam* is shown in Scheme 31. In the first biotransformation step, the SCH$_3$ group of brassicanal A was oxidized to the corresponding sulfoxide (106). In subsequent steps the aldehyde group of 52 was reduced to the alcohol (107) and then further to the 3-methylindole (108).

**Scheme 31**

\[\text{CHO} \quad \text{CHO} \]
\[
\begin{align*}
\text{SCH}_3 & \quad \text{SCH}_3 \\
\text{H} & \quad \text{H} \\
52 & \quad 106
\end{align*}
\]

\[\begin{align*}
\text{CH}_3 & \quad \text{CH}_2\text{OH} \\
\text{O} & \quad \text{O} \\
108 & \quad 107
\end{align*}\]
Studies on the biotransformation of the phytoalexin brassilexin (29) by the blackleg fungus indicated that the phytoalexin is transformed but no biotransformation products were detected or isolated (Pedras et al., 1997). This confirmed the results of a previous study on fungal metabolism of brassilexin (Rouzel et al., 1995). Possible metabolic products of brassilexin might be very polar and more soluble in the aqueous medium than in organic solvents, thus precluding extraction and detection.

The cruciferous phytoalexin camalexin (38) was found to be stable in the culture medium under the incubation conditions with the blackleg fungus. None of the blackleg fungal isolates tested metabolized camalexin or appeared to be affected by a concentration of 5 x 10^{-4} M (Pedras et al., 1997). Camalexin was, however, metabolized by R. solani, a pathogen of Camelina sativa (false flax), a plant that produces camalexin. Incubation of R. solani with camalexin resulted in the detection and isolation of three metabolites (Pedras and Khan, 1997). Their structures were deduced to be 109, 110, and 111 from their respective spectroscopic data in comparison with camalexin, and confirmed by synthesis. Further biotransformation of metabolites 109, 110 and 111 led to establishment of the biotransformation sequence shown in Scheme 32. The first biotransformation step of camalexin is a simple oxidation of its indole ring to yield 109. The next transformation step led to oxidative bond cleavage of the indole ring, yielding compound 111. This was the first time that oxidation of the indole ring of a cruciferous phytoalexin had been observed (Pedras and Khan, 1997). Previous work with other cruciferous phytoalexins indicated that fungal oxidation occurred at the indole substituents at C-2 or C-3. Metabolite 110 could arise from degradation of 5-hydroxycamalexin (109) through elimination of sulfur and acetylene.
The biotransformation products of camalexin by \textit{R. solani} were found to be significantly less toxic than camalexin. While agar plates containing camalexin at $5 \times 10^{-4}$ M inoculated with \textit{R. solani} showed no mycelial growth after incubation for one week, metabolites 109, 110, and 111 at identical concentration had only a very slight inhibitory effect (Pedras and Khan, 1997). This led to the conclusion that the metabolism of camalexin by \textit{R. solani} is a detoxification process that allows the pathogen to overcome a plant's chemical defense.

The results of studies on the biotransformation of cruciferous phytoalexins have demonstrated that the virulent blackleg fungus may utilize different enzymes to transform
diverse phytoalexins. Compared to brassinin, the biotransformation pathway for brassicanal A, for example, was significantly different. The transformation of brassicanal A and brassilexin by virulent isolates of *P. lingam* was also found to be slower than that of the phytoalexin brassinin (Pedras and Khan, 1996).

A correlation between the bioactivity of the phytoalexins and of their biotransformation products will allow an understanding of the detoxification mechanisms employed by the fungus to overcome the plant's defenses. It should then be possible to biorationally design antifungal agents selective against the fungal pathogen (Pedras and Khan, 1996).
CHAPTER 2: RESULTS AND DISCUSSION

2.1 Methyl tryptamine dithiocarbamate

This section describes and discusses the results obtained from the metabolism of methyl tryptamine dithiocarbamate (3) by the fungal pathogens *P. lingam*, and *A. brassicae*. Methyl tryptamine dithiocarbamate (3), a homologue of the cruciferous phytoalexin brassinin (1) was chosen so as to investigate the effect of extending the side chain of the indole ring on the metabolism by fungal pathogens and antifungal activity. Previous studies (Pedras and Taylor, 1991, 1993; Pedras et al., 1992) had shown that the metabolism of brassinin (1) by *P. lingam* isolates occurred in the side chain.

2.1.1 Synthesis and characterization of methyl tryptamine dithiocarbamate

Methyl tryptamine dithiocarbamate (3) was synthesized from tryptamine (112) as outlined in Scheme 33 and described in the experimental section.

Scheme 33

![Scheme 33](image)

Reagents: (a) CS₂, Et₃N, py; MeI.
This procedure is the same as that employed for the synthesis of brassinin from indole-3-methanamine (21) (Scheme 2 in section 1.4.1.1) (Takasugi et al., 1988). The $^1$H NMR spectrum showed the signals expected for a thiomethyl group at $\delta$ 2.56 (s, 3H), two methylene groups at $\delta$ 3.11 (m, 2H) and 4.06 (m, 2H), five aromatic protons characteristic of a 3-substituted indole ring, and two exchangeable protons at $\delta$ 8.07 (br s 1H, indolic NH) and 7.0 (br s, 1H, side chain NH). The molecular formula was determined to be $C_{12}H_{14}N_{2}S_{2}$ by HREIMS ($m/z$ found 250.0600 M$^+$, calcd. 250.0598) in combination with $^1$H and $^{13}$C NMR data. The $^{13}$C NMR spectrum showed 12 signals corresponding to 12 carbon atoms. Eight signals for aromatic carbons were characteristic of the 3-substituted indole ring. One signal at $\delta$ 198.8 (s) indicated the presence of a thiocarbonyl group. The remaining three signals at $\delta$ 47.2 (t), 24.0 (t) and 18.1(q) were those two methylene groups and one methyl group, respectively. The FTIR spectrum showed characteristic absorption bands at 3408, 3324 cm$^{-1}$ for NH groups.

2.1.2 Biotransformation studies

In order to determine the optimum period for isolation of metabolites time-course experiments were conducted as detailed in the experimental section. A preliminary study of the biotransformation of methyl tryptamine dithiocarbamate (3) by ENG-53, a virulent isolate of $P$. lingam was undertaken, alongside that of the phytoalexin brassinin (1). The latter was shown to be metabolized (Pedras and Taylor, 1991, 1993; Pedras et al., 1992) by the blackleg fungus. In this study methyl tryptamine dithiocarbamate at two concentrations (final solution concentrations $2 \times 10^{-4}$ M and $4 \times 10^{-4}$ M) in DMSO were administered to fungal cultures and uninoculated media. Similarly brassinin (final concentration $4 \times 10^{-4}$ M) solutions in DMSO were added to fungal cultures and uninoculated media.

Brassinin (1) was biotransformed after 4-6 days to yield the metabolites previously reported (Pedras and Taylor, 1991, 1993; Pedras et al., 1992). The HPLC
chromatograms of methyl tryptamine dithiocarbamate at the lower concentration ($2 \times 10^{-4}$ M) indicated the disappearance of the methyl tryptamine dithiocarbamate peak and the emergence of new peaks not present in the control samples. No biotransformation took place in fungal cultures incubated with the higher concentration ($4 \times 10^{-4}$ M) of the compound. The dithiocarbamate (3) completely inhibited fungal growth at this concentration. The results of this preliminary experiment established that methyl tryptamine dithiocarbamate was biotransformed by the blackleg fungus and that it inhibited fungal growth at concentrations of $4 \times 10^{-4}$ M. Subsequent biotransformation experiments were conducted at concentrations of $2 \times 10^{-4}$ M or lower.

In order to isolate and characterize the metabolites of methyl tryptamine dithiocarbamate biotransformation the process was scaled up. The scaled up experiments were conducted in a similar manner as for the time-course ones except for the larger number of culture flasks employed. The processing of samples for the time-course and scale up experiments is described in the experimental section.

2.1.2.1 Metabolism of methyl tryptamine dithiocarbamate by Phoma lingam virulent isolates

The time-course experiments established that methyl tryptamine dithiocarbamate (3) was metabolized by P. lingam virulent isolates BJ-125 and ENG-53. In a series of five scale up experiments the metabolites of methyl tryptamine dithiocarbamate biotransformation were isolated and characterized. The metabolites were isolated by flash column chromatography and / or preparative TLC and characterized by spectroscopic techniques (FTIR, $^1$H NMR, $^{13}$C NMR, MS). The biotransformation resulted in the isolation and characterization of eight metabolites which were identified as tryptamine (112), $N_e$-acetyltryptamine (113), methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (114), methyl tryptamine dithiocarbamate-S-oxide (115), methyl 2-oxotryptamine dithiocarbamate (116), indole-3-acetic acid (117), methyl indole-
3-acetate (118) and oxindole-3-acetic acid (119). Isolated metabolites accounted for ca. 70-80% of the original amount of 3 after 14 days incubation. The structures of these metabolites are shown in Scheme 34. The R_f values and HPLC peak retention times in minutes are shown in Table 2.1.

**Table 2.1** HPLC peak retention times and R_f values for methyl tryptamine dithiocarbamate (3) and products of biotransformation by *P. lingam*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R_f value</th>
<th>HPLC peak retention time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl tryptamine dithiocarbamate (3)</td>
<td>0.7</td>
<td>20.4</td>
</tr>
<tr>
<td>Tryptamine (112)</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>N_b-Acetyltryptamine (113)</td>
<td>0.2</td>
<td>6.6</td>
</tr>
<tr>
<td>Methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (114)</td>
<td>0.5</td>
<td>14.5</td>
</tr>
<tr>
<td>Methyl tryptamine dithiocarbamate-S-oxide (115)</td>
<td>0.1</td>
<td>11.6 (br)</td>
</tr>
<tr>
<td>Methyl 2-oxotryptamine dithiocarbamate (116)</td>
<td>0.3</td>
<td>12.5</td>
</tr>
<tr>
<td>Indole-3-acetic acid (117)</td>
<td>0.5</td>
<td>2.8 (br, variable)</td>
</tr>
<tr>
<td>Methyl indole-3-acetate (118)</td>
<td>0.7</td>
<td>13.2</td>
</tr>
<tr>
<td>Oxindole-3-acetic acid (119)</td>
<td>0.2</td>
<td>1.7</td>
</tr>
<tr>
<td>Methyl 3-hydroxy-2-oxotryptamine dithiocarbamate (120)</td>
<td>0.2</td>
<td>7.6</td>
</tr>
<tr>
<td>Tryptophol (121)</td>
<td>0.3</td>
<td>6.9</td>
</tr>
</tbody>
</table>

^R_f values in CH_2Cl_2-MeOH (95 : 5) except for indole-3-acetic acid, oxindole-3-acetic acid in CH_2Cl_2-MeOH (90 : 10) and tryptamine in CH_2Cl_2-MeOH-NH_4OH (80 : 20 : 1).
Scheme 34

\[ \text{[Chemical Structures]} \]

\( ^a \text{Pathway for biotransformation of methyl tryptamine dithiocarbamate (3) by virulent } P. \) 
\( \text{lingam.} \)

In order to establish the sequence of the biotransformation steps and to map out the pathways, each of the metabolites tryptamine (112), \( N \)-acetyltryptamine (113) methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-\( b \)]indol-1(2H)-yl carbodithioate (114),
methyl tryptamine dithiocarbamate-S-oxide (115), methyl 2-oxotryptamine dithiocarbamate (116), indole-3-acetic acid (117), methyl indole-3-acetate (118) and oxindole-3-acetic acid (119) was subjected to further biotransformation by virulent isolates of *P. lingam*. If further biotransformation led to detection of new compounds, i.e. not detected in the biotransformation of methyl tryptamine dithiocarbamate, scale up experiments were carried out to isolate and characterize them.

Methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (114), indole-3-acetic acid (117), and oxindole-3-acetic acid (119) underwent no further biotransformation on incubation with the fungus for up to 14 days. Tryptamine (112) was rapidly (within 24 hours) biotransformed to give N<sub>b</sub>-acetyltryptamine and indole-3-acetic acid. N<sub>b</sub>-acetyltryptamine (113) was partially (ca. 50%) transformed to indole-3-acetic acid after 14 days of incubation. Methyl indole-3-acetate (118) was biotransformed (within 48 hours) into indole-3-acetic acid. Methyl tryptamine dithiocarbamate-S-oxide (115) was reduced (within 6 hours) to methyl tryptamine dithiocarbamate (3). Methyl 2-oxotryptamine dithiocarbamate (116) yielded an apparently new metabolite with HPLC peak retention time of 7.6 minutes. In a series of scale up experiments this metabolite was isolated, characterized and identified as methyl 3-hydroxy-2-oxotryptamine dithiocarbamate (120). Its *R*<sub>f</sub> value and HPLC peak retention time are shown in Table 2.1. In control experiments, each of the metabolites was stable in the cultures up to 14 days of incubation.

From the results of the biotransformation of methyl tryptamine dithiocarbamate and its metabolites by *P. lingam* virulent isolates, the pathways for biotransformation shown in Scheme 34 are proposed. These fungal isolates biotransformed methyl tryptamine dithiocarbamate (3) via three distinct pathways. The major pathways led to methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (114) and to indole-3-acetic acid (117) via tryptamine (112) and N<sub>b</sub>-acetyltryptamine (113). The minor pathway led to methyl 2-oxotryptamine dithiocarbamate (116).
2.1.2.2 Metabolism of methyl tryptamine dithiocarbamate by Phoma 
lingam avirulent isolates

As shown by the time-course experiments, methyl tryptamine dithiocarbamate (3) was biotransformed by avirulent P. lingam isolate Unity. In a scale up procedure the metabolites of the biotransformation of methyl tryptamine dithiocarbamate were isolated and characterized in a similar manner as for the virulent isolates. The biotransformation resulted in the isolation, characterization and identification of five metabolites as tryptamine (112), N₆-acetyltryptamine (113), indole-3-acetic acid (117), tryptophol (121) and indole-3-carboxylic acid (104). The total amount of isolated metabolites was ca. 70% of the initial amount of 3 after 4 days incubation. The first three metabolites were also isolated from biotransformations with virulent isolates (Scheme 34). The structures of the latter two metabolites are given in Scheme 35. The Rₜ values, and HPLC retention times in minutes were as given in Table 2.1.

In order to establish the sequence of the biotransformation steps and hence the biotransformation pathway, each of the metabolites tryptamine (112), N₆-acetyltryptamine (113), indole-3-acetic acid (117), tryptophol (121) and indole-3-carboxylic acid (104) was subjected to further incubation with P. lingam isolate Unity. Indole-3-carboxylic acid (104) underwent no further transformation and was recovered unchanged from the cultures after 14 days. Tryptamine (112) was further biotransformed to give tryptophol and indole-3-carboxylic acid. N₆-acetyltryptamine (113) yielded tryptophol and indole-3-carboxylic acid on further biotransformation by the fungus. Indole-3-acetic acid (117) was transformed to yield indole-3-carboxylic acid. Tryptophol (121) was partially (ca. 80%) transformed to indole-3-carboxylic acid after 14 days incubation. In control experiments each of the compounds was stable in the cultures for at least 14 days. From the results of biotransformation of methyl tryptamine dithiocarbamate and metabolites by P. lingam avirulent isolates the pathway for biotransformation shown in Scheme 35 is
proposed. The avirulent isolate Unity biotransformed methyl tryptamine dithiocarbamate (3) through one major pathway to yield indole-3-carboxylic acid (104) via tryptamine (112) and N$_6$-acetyltryptamine (113).

**Scheme 35**

- **3**
- **112** $R = H$
- **113** $R = C O C H _ 3$
- **104**
- **117**
- **121**

*Pathway for biotransformation of methyl tryptamine dithiocarbamate (3) by avirulent *P. lingam*.*
2.1.2.3 Metabolism of methyl tryptamine dithiocarbamate by Alternaria brassicae

Time-course experiments showed that methyl tryptamine dithiocarbamate was biotransformed by A. brassicae isolates AB3 and IBJ to yield two metabolites. These metabolites were isolated, characterized and identified as tryptamine (112) and N₆-acetyltryptamine (113).

2.1.3 Characterization and identity of methyl tryptamine dithiocarbamate metabolites

Tryptamine (112) was found to have the molecular formula C₁₀H₁₂N₂ by HREIMS (m/z found 160.1004 M⁺, calcd. 160.1000) in combination with ¹H and ¹³C NMR spectral data. From the ¹H and ¹³C NMR spectral data a 3-substituted indole structural moiety was deduced. In addition there were signals for two methylene groups at δₗ 2.79, (m, 4H); δₛ 42.8 (t), 29.6 (t) and three D₂O exchangeable protons at δ 10.85 (br s, 1H); 1.60 (br s, 2H). The FTIR showed characteristic absorption bands at 3404, 3345 cm⁻¹ (NH). Compared to methyl tryptamine dithiocarbamate this compound lacked signals corresponding to thiomethyl and thiocarbonyl groups. On the basis of the spectroscopic data the compound was identified as tryptamine (112). The identity was further corroborated by comparison of the physical data with those of an authentic sample of tryptamine available commercially from Aldrich Chemical Co.

N₆-acetyltryptamine (113) had a molecular formula C₁₂H₁₄N₂O as determined by HREIMS (m/z found 202.1105, calcd. 202.1106) in combination with ¹H and ¹³C NMR spectral data. The ¹H and ¹³C NMR spectroscopic data were similar to those of methyl tryptamine dithiocarbamate, suggesting that it also possessed an indole skeleton substituted at the C-3 position. In the ¹H and ¹³C NMR spectral data, the major differences were the presence of signals for a methyl at δₗ 1.90 (s, 3H), δₛ 23.2 (q) characteristic of an acetyl group and a carbonyl carbon at δ 170.4 (s) instead of the signals corresponding
to the thiomethyl and thiocarbonyl groups. To account for the molecular formula (C_{12}H_{14}N_{2}O) together with \textsuperscript{13}C NMR data the carbonyl carbon should be assigned to an amide, and this was supported by the observation of FTIR absorption band at 1653 cm\textsuperscript{-1}. These spectral data suggested that the dithiocarbamate group of methyl tryptamine dithiocarbamate had been replaced by an acetamide group in this compound. This conclusion led to the identification of the metabolite as N\textsubscript{b}-acetyltryptamine (113). The identity of the compound as N\textsubscript{b}-acetyltryptamine was confirmed by comparison of physical data (TLC, \textsuperscript{1}H and \textsuperscript{13}C NMR) with those of an authentic sample of N\textsubscript{b}-acetyltryptamine synthesized from tryptamine as described in experimental section.

Methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (114) was found to be optically active ([\alpha]_D = +473, c 0.0014, CH\textsubscript{2}Cl\textsubscript{2}). The EIMS showed a molecular ion peak at \textit{m} / \textit{z} 266, C\textsubscript{12}H\textsubscript{14}N\textsubscript{2}OS\textsubscript{2} (HREIMS, \textit{m} / \textit{z} found 266.0547 M\textsuperscript{+}, calcld. 266.0548), one oxygen unit difference from methyl tryptamine dithiocarbamate. The FTIR, \textit{v}_\textit{max} 3368 (NH), 1610, 1415 cm\textsuperscript{-1} (aromatic C=C) and \textsuperscript{13}C NMR, \delta 87.2 (d), 86.2 (s) spectra suggested the presence of an indoline rather than an indole moiety. The \textsuperscript{1}H NMR showed signals for four consecutively coupled aromatic protons, a heteroatom substituted methine proton at \delta 5.71 (s, 1H), four coupled aliphatic protons, and a singlet for the thiomethyl protons at \delta 2.62 (s, 3H). Compared to methyl tryptamine dithiocarbamate, the aromatic singlet due to H-2 (\delta 6.99) was replaced by a signal at \delta 5.71 (s), indicating that the double bond at the 2, 3-position was modified. Four aliphatic protons were found to have the coupling pattern of nonmagnetically equivalent methylene protons, especially H\textsubscript{2}-2 (\delta 3.28-3.37 and 4.06-4.14). The four aromatic, and four aliphatic protons (\delta 3.28-3.37 and 4.06-4.14 coupled to \delta 2.52-2.59) were further correlated by \textsuperscript{1}H-\textsuperscript{1}H COSY. Additional HMQC and HMBC experiments suggested the presence of a pyrroloindole system. From the spectroscopic data, the structure was determined to be methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (114). The absolute configuration of 114 is yet to be
determined. The identity of metabolite 114 was further corroborated by synthesis. Photosensitized oxidation of methyl tryptamine dithiocarbamate (3) yielded 114. This preparation followed a procedure previously reported for the corresponding photosensitized oxidation of methyl tryptamine carbamate (Nakagawa et al., 1977). Enzymatic oxidative cyclization via the epoxide (122) (Scheme 36) (Pedras and Okanga, 1998b) could account for the formation of this compound from methyl tryptamine dithiocarbamate (3).

Methyl tryptamine dithiocarbamate-S-oxide (115) had a molecular formula C_{12}H_{14}N_{2}OS_{2} as determined by HREIMS (m/z found 266.0559 M⁺, calcd. 266.0548). This formally represents addition of one oxygen unit to methyl tryptamine
dithiocarbamate. The $^1$H NMR spectrum displayed resonances comparable to those of methyl tryptamine dithiocarbamate (within 0.1 ppm), except for the thiomethyl ($\delta$ 2.35 vs 2.56 for methyl tryptamine dithiocarbamate) and -CH$_2$N ($\delta$ 3.80 vs 4.06 for methyl tryptamine dithiocarbamate) groups. Furthermore, the $^{13}$C NMR spectrum was similar to that of methyl tryptamine dithiocarbamate (within 2 ppm) except for resonances attributable to thiocarbonyl ($\delta_C$ 194.5 vs 198.8 in methyl tryptamine dithiocarbamate) and -SCH$_3$ ($\delta_C$ 13.3 vs 18.1 in methyl tryptamine dithiocarbamate) groups. These results and the FTIR spectrum ($\nu_{\text{max}}$ 905 cm$^{-1}$) indicated the presence of an S-oxide group. On the basis of the spectroscopic data the compound was identified as methyl tryptamine dithiocarbamate-S-oxide (115). Identity was further confirmed by synthesis of the compound from methyl tryptamine dithiocarbamate by oxidation with m-CPBA as described in the experimental section.

Methyl 2-oxotryptamine dithiocarbamate (116) was found to be optically active ([\alpha]_D = -50.0, c 0.008, CH$_2$Cl$_2$). The molecular formula was determined to be C$_{12}$H$_{14}$N$_2$OS$_2$ on the basis of HREIMS ($m / z$ found 266.0550, calcd. 266.0548) in combination with $^1$H and $^{13}$C NMR data. Compared to methyl tryptamine dithiocarbamate this compound also had one additional oxygen unit. The $^1$H NMR spectrum displayed signals for four coupled aromatic protons, a five-multiplet spin system for aliphatic protons (H-3 and -CH$_2$CH$_2$N), a singlet for thiomethyl protons at $\delta$ 2.59 (s, 3H) and two exchangeable protons at $\delta$ 8.57 (br s, 1H, indolic NH) and 8.02 (br s, 1H, side chain NH). Signals for four aliphatic protons at $\delta$ 4.04-4.10 (m, 1H) and 3.55-3.59 (m, 1H); 2.38-2.45 (m, 1H) and 2.09-2.18 (m, 1H) had the coupling patterns of nonmagnetically equivalent methylene protons. Compared to methyl tryptamine dithiocarbamate, the aromatic singlet due to H-2 ($\delta$ 6.99) was replaced with a new signal at $\delta$ 3.84-3.88. Also there was a significant upfield shift for the H$_2$-1' methylene protons ($\delta$ 2.38-2.45 and 2.09-2.18 vs 3.08-3.12 in methyl tryptamine dithiocarbamate). The $^{13}$C NMR spectrum showed 12 signals for six aromatic carbons at $\delta$ 140.9 (s), 128.9 (s), 128.4 (s), 124.1
(d), 123.0 (d), and 110.1 (d), a carbonyl carbon at δ 180.7 (s), a thio carbonyl carbon at δ 199.0 (s) and four aliphatic carbons at δ 45.4 (t), 45.0 (d), 28.7 (t) and 18.0 (q). Compared with methyl tryptamine dithiocarbamate, the signals for two aromatic carbons at δ 112.3 (s) and 122.5 (d) were replaced by signals for an aliphatic carbon and a carbonyl carbon at δ 45.0 (d) and 180.7 (s), respectively. The spectral data again indicated that the double bond at the 2, 3-positions was modified. The four coupled aromatic and five coupled aliphatic protons were further correlated by spin decoupling and 2D-NMR spectral data. Irradiation of the multiplets at δ 4.04-4.10 and 3.55-3.59 (H$_2$-2') affected the multiplets at δ 3.84-3.88 (H-3) as well as those at δ 2.38-2.45 and 2.09-2.18 (H$_2$-1'). Similarly, irradiation of the multiplets at δ 2.38-2.45 and 2.09-2.18 (H$_2$-1') affected the multiplets at δ 4.04-4.10 and 3.55-3.59 (H$_2$-2') as well as those at δ 3.84-3.88 (H-3). Irradiation at δ 3.84-3.88 (H-3) only affected the multiplets at δ 2.38-2.45 and 2.09-2.18 (H$_2$-1'). The HMQC and HMBC spectra showed the expected correlation of a 2-oxo-3-ethylindole. On the basis of the spectroscopic data the structure of this compound was determined to be methyl 2-oxotryptamine dithiocarbamate (116). The absolute configuration of metabolite 116 has not been determined. Identity was further corroborated by synthesis of the compound from methyl tryptamine dithiocarbamate-S-oxide. Exposure of methyl tryptamine dithiocarbamate-S-oxide to deuterated chloroform at room temperature or acidification with p-TsOH gave compound (116). This transformation was based on the observation that methyl tryptamine dithiocarbamate-S-oxide rearranged on standing in CDCl$_3$ at room temperature. In the course of characterizing the S-oxide it happened that the sample was left in the NMR tube at room temperature for 12 hours. After this period of time a $^1$H NMR spectrum obtained on the same sample indicated the presence of two different compounds. Separation of this mixture on TLC yielded methyl 2-oxotryptamine dithiocarbamate (40%) together with methyl tryptamine dithiocarbamate (20%) and polar unidentified products. In a one-pot synthesis, oxidation of methyl tryptamine dithiocarbamate with m-CPBA, followed by
acidification of the reaction mixture with $p$-TsOH yielded methyl 2-oxotryptamine dithiocarbamate directly. These results suggested that formation of methyl 2-oxotryptamine dithiocarbamate (116) from methyl tryptamine dithiocarbamate (3) might be initiated by protonation of the indole ring at C-3, followed by intramolecular oxygen transfer from C=S=O to C-2 with regeneration of the dithiocarbamate group as shown in Scheme 37 (Pedras and Okanga, 1998b).

Scheme 37

![Scheme 37](attachment:image.png)

Attempted synthesis of this compound from tryptamine via oxotryptamine (123) (Scheme 38) was unsuccessful. Treatment of tryptamine with DMSO-HCl (1 : 2) (Szabó and Szabó, 1979) only yielded tryptamine hydrochloride and none of the required 2-oxotryptamine hydrochloride. Oxotryptamine (123), is known to be unstable as a free
base. It is reported that attempts to synthesize this compound in free form met with no success and gave a complicated mixture of unidentified products (Nakagawa et al., 1980).

Scheme 38

\[ \text{Scheme 38} \]

\[ \text{112} \]

\[ \text{a} \] \text{DMSO} / \text{HCl}; \text{b} \] \text{CS}_2, \text{Et}_3\text{N}, \text{py}; \text{MeI}.

The instability of oxotryptamine has been attributed to its transformation to 3-(O-aminophenyl)-2-pyrrolidone (126) (Scheme 39) by intramolecular acyl migration via the intermediate 125.

Indole-3-acetic acid (117) was found to have the molecular formula $\text{C}_{10}\text{H}_9\text{NO}_2$ by HREIMS ($m/z$ found 175.0631 ($M^+$, calcd. 175.0633). The aromatic region in both the $^1\text{H}$ NMR and $^{13}\text{C}$ NMR spectra was similar to that of methyl tryptamine dithiocarbamate, indicating that it also possessed an indole ring substituted at C-3. The $^1\text{H}$ and $^{13}\text{C}$ NMR spectra showed signals at $\delta_\text{H} 3.62$, (s, 2H), 12.18 (br s, 1H); $\delta_\text{C} 31.0$ (t), 173.2 (s)
indicating the presence of one methylene and a carboxylic acid group. The EIMS showed a base peak at \( m/z \) 130 and the FTIR spectrum showed characteristic bands at 2500-3300 (OH), 3391 (NH) and 1700 cm\(^{-1}\)(-C=O). On the basis of these spectral data the compound was identified as indole-3-acetic acid (117). This was further confirmed by comparison of physical data (TLC, \(^1\)H and \(^{13}\)C NMR, FTIR, MS) with those of an authentic sample of indole-3-acetic acid commercially available from Aldrich Chemical Co.

Scheme 39

![Scheme 39](image)

Methyl indole-3-acetate (118) had a formula \( \text{C}_{11}\text{H}_{11}\text{NO}_2 \) as established on the basis of HREIMS (\( m/z \) found 189.0788 M\(^+\), calcd. 189.0790). The \(^1\)H and \(^{13}\)C NMR data were similar to those of indole-3-acetic acid. The \(^1\)H NMR spectrum showed signals for
five aromatic protons characteristic of a C-3 substituted indole skeleton. Compared with indole-3-acetic acid the major difference was the observation of a methoxy signal at δ 3.70, (s, 3H) instead of the signal corresponding to the carboxyl proton. To account for the molecular formula (C_{11}H_{11}NO_{2}) together with {sup 13}C NMR data, the carbonyl carbon signal at δ 172.6 (s) should be assigned to an ester, and this was supported by the observation of FTIR absorption at δ 1733 cm^{-1}. These spectral data suggested that the carboxyl group of indole-3-acetic acid had been converted to a methyl ester. This led to identity of this compound as methyl indole-3-acetate (118). The identity was further confirmed by comparison of physical data (TLC, NMR, FTIR) with those of an authentic sample obtained by methylation of indole-3-acetic acid with diazomethane.

Oxindole-3-acetic acid (119) had the molecular formula C_{10}H_{9}NO_{3} as determined by HREIMS (m/z found 191.0577 M^{+}, calcd. 191.0582). The FTIR spectrum exhibited characteristic absorption bands at 2500-3500 (NH, OH), and 1700 cm^{-1} (-C=O). The {sup 1}H NMR showed signals for four coupled aromatic protons, three aliphatic protons of a methine coupled to a methylene forming an ABX spin system. In addition, there were signals for two D_{2}O exchangeable protons at δ 12.34 (br s, COOH) and 10.39 (s, indolic NH). The {sup 13}C NMR spectrum clearly showed the signals of 10 carbons: two carbonyl carbons at δ 178.1 (s), 172.1 (s), six aromatic carbons at δ 142.8 (s), 129.3 (s), 127.6 (d), 123.6 (d), 121.1 (d), 109.1 (d), one methine carbon at δ 41.8 (d) and one methylene carbon at δ 34.0 (t). These spectroscopic data indicated this compound to be oxindole-3-acetic acid (119). The identity was further established and confirmed by synthesis from indole-3-acetic acid. The latter compound was oxidized with DMSO / HCl (Szabό and Szabό, 1979) as described in the experimental section. Although a similar reaction could explain the presence of oxindole-3-acetic acid in fungal cultures incubated with methyl tryptamine dithiocarbamate, control experiments indicated that such a process did not occur under the conditions utilized for fungal metabolism of methyl tryptamine dithiocarbamate.
Methyl 3-hydroxy-2-oxotryptamine dithiocarbamate (120) was found to have the molecular formula C_{12}H_{14}N_{2}O_{5}S_{2} by HREIMS (m / z found 282.0497 M⁺, calcd. 282.0497) in combination with ¹H and ¹³C NMR spectral data. The ¹H and ¹³C NMR spectral data were similar to those of methyl 2-oxotryptamine dithiocarbamate. The major difference observed was the presence of a quaternary carbon signal at δ 76.5 (s) in the ¹³C NMR spectrum of this compound instead of a methine carbon signal at ca. δ 45.4 (d). The low field chemical shift of this quaternary carbon suggested attachment to a hydroxyl group. In the ¹H NMR spectrum there was no signal corresponding to a C-3 methine proton as observed for methyl 2-oxotryptamine dithiocarbamate. The FTIR spectrum showed characteristic absorption bands at 3305, 3271 (NH, OH) and 1715 cm⁻¹ (C = O). On the basis of these spectroscopic data the compound was identified as methyl 3-hydroxy-2-oxotryptamine dithiocarbamate (120).

Tryptophol (121) was determined to have the molecular formula C_{10}H_{11}NO by HREIMS (m / z found 161.0841 M⁺, calcd. 161.0841). The FTIR spectrum showed characteristic absorption bands at 3398, 3316, cm⁻¹ (OH, NH). The ¹H NMR spectrum displayed signals for five aromatic protons characteristic of a 3-substituted indole ring, and two methylene groups. In addition, there were two signals for two D₂O exchangeable protons at δ 8.04 (br s, 1H, indolic NH), 1.85 (br s, 1H, OH). The ¹³C NMR spectrum showed 10 signals corresponding to 10 carbon atoms: eight aromatic and two aliphatic. The signal at δ 62.6 (t) corresponding to one of the methylene carbons was significantly shifted downfield compared to that of methyl tryptamine dithiocarbamate. This downfield shift indicated attachment to oxygen rather than nitrogen. On the basis of these spectroscopic data the compound was identified as tryptophol (121). Identity was confirmed by comparison of the physical data (TLC, NMR, FTIR) with those of an authentic sample of tryptophol available commercially from Aldrich Chemical Co.

Indole-3-carboxylic acid (104) was found to have the molecular formula C₉H₇NO₂ on the basis of HREIMS (m / z found 161.0479 M⁺, calcd. 161.0477) in combination
with $^1$H and $^{13}$C NMR spectral data. The $^1$H and $^{13}$C NMR spectral data revealed the presence of a 3-substituted indole structural unit. Compared to methyl tryptamine dithiocarbamate, the major differences were that this compound showed a signal at $\delta_\text{H}$ 12.07, (br s, 1H); $\delta_\text{C}$ 166.0 (s) for a carboxylic acid group and no signals attributable to methylenes, thiomethyl and thiocarbonyl groups. The FTIR spectrum showed characteristic bands at 3315 (NH), 2400-3500 (OH), 1665 cm$^{-1}$ (C = O). On the basis of these spectroscopic data the compound was identified as indole-3-carboxylic acid (104). Identity was further confirmed by comparison of the physical data with those of an authentic sample of indole-3-carboxylic acid commercially available from Aldrich Chemical Co.

2.1.4 Antifungal activity of methyl tryptamine dithiocarbamate

2.1.4.1 Bioassay of methyl tryptamine dithiocarbamate and brassinin

Initially, the antifungal activity of methyl tryptamine dithiocarbamate (3) and the phytoalexin brassinin (1) were assayed. The purpose of these initial bioassays was to compare the activity of methyl tryptamine dithiocarbamate with that of brassinin. The results of these bioassays would also allow the determination of the concentrations of methyl tryptamine dithiocarbamate to be used in biotransformation experiments. These compounds at two concentrations ($1 \times 10^{-4}$ M and $5 \times 10^{-4}$ M) were bioassayed against P. lingam isolates BJ-125 (virulent) and Unity (avirulent). The results are presented in Tables 2.2 and 2.3.

These results indicated that methyl tryptamine dithiocarbamate was more toxic to P. lingam isolates than brassinin. In solid agar incorporating brassinin at a concentration of $5 \times 10^{-4}$ M P. lingam spores generally took 72-96 hours to germinate. Bioassays with methyl tryptamine dithiocarbamate at the same concentration in solid agar indicated that the spores germinated after 96-120 hours. The difference in antifungal activity between
methyl tryptamine dithiocarbamate and brassinin was more distinct in liquid cultures. At a concentration of $5 \times 10^{-4}$ M methyl tryptamine dithiocarbamate completely inhibited mycelial growth while brassinin showed only partial inhibition.

**Table 2.2** Bioassay of brassinin (1) and methyl tryptamine dithiocarbamate (3) against *P. lingam* isolate BJ-125 after 72 hours incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>% Inhibition $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brassinin (1)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>100</td>
</tr>
<tr>
<td>Methyl tryptamine dithiocarbamate (3)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>100</td>
</tr>
</tbody>
</table>

$^a$ % inhibition = 100 - [(growth in treated / growth in control) $\times$ 100]; results are the mean of at least three separate experiments, standard deviation $\pm$ 0.5

**Table 2.3** Bioassay of brassinin (1) and methyl tryptamine dithiocarbamate (3) against *P. lingam* isolate Unity after 72 hours incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>% Inhibition $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brassinin (1)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>34</td>
</tr>
<tr>
<td>Methyl tryptamine dithiocarbamate (3)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>100</td>
</tr>
</tbody>
</table>

$^a$ % inhibition = 100 - [(growth in treated / growth in control) $\times$ 100]; results are the mean of at least three separate experiments, standard deviation $\pm$ 0.5

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2.1.4.2 Bioassay of methyl tryptamine dithiocarbamate and metabolites

Methyl tryptamine dithiocarbamate and each of its biotransformation products were bioassayed at two concentrations of $1 \times 10^{-4}$ M and $5 \times 10^{-4}$ M against \textit{P. lingam} (virulent BJ-125 or avirulent Unity) as well as \textit{A. brassicae} isolates. The purpose of these assays was to compare the antifungal activity of the metabolites with the parent compound and to determine if the biotransformation is a detoxification process. The results of these bioassays are shown in Tables 2.4, 2.5 and 2.6. These results indicate that the biotransformation of methyl tryptamine dithiocarbamate by virulent or avirulent isolates of \textit{P. lingam} as well as \textit{A. brassicae} are detoxification processes. Each of the metabolites was found to be much less inhibitory to spore germination and radial mycelial growth of each fungus than the parent compound.

2.1.5 Effect of methyl tryptamine dithiocarbamate on phytotoxin production

As discussed in section 1.3.1, virulent isolates of \textit{P. lingam} produce the host-selective phytotoxin phomalide (7) as well as nonselective toxins in liquid cultures. The phytoalexin brassinin was shown to inhibit the biosynthesis of the nonselective phytotoxins but not phomalide by virulent \textit{P. lingam} isolates (Pedras and Taylor, 1993). Therefore it was important to determine the effect of methyl tryptamine dithiocarbamate on the production of phytotoxins.

In cultures incubated with methyl tryptamine dithiocarbamate no nonselective phytotoxins were detected by HPLC until the dithiocarbamate was completely metabolized by the fungus. After complete metabolism of the compound, the virulent isolates of \textit{P. lingam} produced the nonselective toxins. The effect of methyl tryptamine dithiocarbamate on the production of phomalide (7) was not observed as the compound was added to 48 h-old cultures when the fungus was already producing the phytotoxin.
Table 2.4 Bioassay of methyl tryptamine dithiocarbamate (3) and metabolites against virulent *P. lingam* isolate BJ-125 after 72 hours incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>% Inhibition $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl tryptamine dithiocarbamate (3)</td>
<td>$1 \times 10^4$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^4$ M</td>
<td>100</td>
</tr>
<tr>
<td>Tryptamine (112)</td>
<td>$1 \times 10^4$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^4$ M</td>
<td>1</td>
</tr>
<tr>
<td>$N_b$-acetyltryptamine (113)</td>
<td>$1 \times 10^4$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^4$ M</td>
<td>2</td>
</tr>
<tr>
<td>Methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (114)</td>
<td>$1 \times 10^4$ M</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^4$ M</td>
<td>5</td>
</tr>
<tr>
<td>Methyl tryptamine dithiocarbamate-S-oxide (115)</td>
<td>$1 \times 10^4$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^4$ M</td>
<td>7</td>
</tr>
<tr>
<td>Methyl 2-oxotryptamine dithiocarbamate (116)</td>
<td>$1 \times 10^4$ M</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^4$ M</td>
<td>3</td>
</tr>
<tr>
<td>Indole-3-acetic acid (117)</td>
<td>$1 \times 10^4$ M</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^4$ M</td>
<td>20</td>
</tr>
<tr>
<td>Methyl indole-3-acetate (118)</td>
<td>$1 \times 10^4$ M</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^4$ M</td>
<td>18</td>
</tr>
<tr>
<td>Oxindole-3-acetic acid (119)</td>
<td>$1 \times 10^4$ M</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^4$ M</td>
<td>2</td>
</tr>
</tbody>
</table>

$^a$ % inhibition = 100 - [(growth in treated / growth in control) × 100]; results are the mean of at least three separate experiments, standard deviation ± 0.5
Table 2.5 Bioassay of methyl tryptamine dithiocarbamate (3) and metabolites against avirulent P. lingam isolate Unity after 72 hours incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>% Inhibition a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl tryptamine dithiocarbamate (3)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>100</td>
</tr>
<tr>
<td>Tryptamine (112)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>1</td>
</tr>
<tr>
<td>$N_\text{a}$-acetyltryptamine (113)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>2</td>
</tr>
<tr>
<td>Indole-3-acetic acid (117)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>10</td>
</tr>
<tr>
<td>Tryptophol (121)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td>Indole-3-carboxylic acid (104)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>1</td>
</tr>
</tbody>
</table>

a % inhibition = 100 - [(growth in treated / growth in control) $\times$ 100]; results are the mean of at least three separate experiments, standard deviation $\pm$ 0.5

In biotransformations with A. brassicae, methyl tryptamine dithiocarbamate was observed to inhibit the production of the host-selective phytotoxin destruxin B (9) (Figure 1.3 in section 1.3.3). In cultures incubated with methyl tryptamine dithiocarbamate the production of the toxin did not occur until complete metabolism of the compound by the fungus (HPLC detection).
Table 2.6 Bioassay of methyl tryptamine dithiocarbamate (3) and metabolites against *A. brassicaceae* isolates after 72 hours incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>% Inhibition a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl tryptamine dithiocarbamate (3)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>100</td>
</tr>
<tr>
<td>Tryptamine (112)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td>N$_6$-acetyltryptamine (113)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
</tbody>
</table>

a % inhibition = 100 - [(growth in treated / growth in control) × 100]; results are the mean of at least three separate experiments, standard deviation ± 0.5

2.2 Cyclobrassinin

This section describes and discusses the results of metabolism of the phytoalexin cyclobrassinin (2) by the phytopathogenic fungi *P. lingam* and *R. solani*. Cyclobrassinin (2) is a phytoalexin first isolated from Chinese cabbage (*B. campestris* L. ssp. *pekinensis*) inoculated with the bacterium *Pseudomonas cichorii* (Takasugi et al., 1988).

2.2.1 Synthesis and characterization of cyclobrassinin

Cyclobrassinin (2) was synthesized from gramine via brassinin as outlined in Schemes 3 (Yamada et al., 1993) and 5 (Takasugi et al., 1988) and detailed in the experimental section. Changing the solvent from CH$_2$Cl$_2$ to THF provided a better yield (44%) in the cyclization step from brassinin to cyclobrassinin. The previously reported (Takasugi et al., 1988) yield with CH$_2$Cl$_2$ as solvent was 35%. The molecular formula was determined to be C$_{11}$H$_{10}$N$_2$S$_2$ by HREIMS (*m/z* found 234.0291 M$^+$, calcld. 234.0285) in combination with $^1$H and $^{13}$C NMR spectral data. The $^1$H NMR spectrum
showed signals for four aromatic protons characteristic of a 2, 3-disubstituted indole ring. The spectrum further showed signals for the presence of a thiomethyl at δ 2.55 (s, 3H) and a methylene at δ 5.08 (s, 2H). The tricyclic structure was evident from the presence of nine sp2 carbon atoms in the 13C NMR spectrum, together with eight degrees of unsaturation. The presence of a 4H-1,3-thiazine moiety was shown by the presence of two peaks in the EIMS at m/z 161 (M-C2H3NS)+ and 73 (C2H3NS)+, a C=N-group (δc 152.0; νmax 1600 cm⁻¹) together with the low carbon chemical shift (δ 48.7) of the methylene group. These characteristic features were consistent with those reported for the compound (Takasugi et al., 1988).

2.2.2 Biotransformation studies

In the biotransformation of cyclobrassinin by R. solani, virulent and avirulent P. lingam isolates, typical time-course procedures were carried out as described in the experimental section. In order to isolate and characterize the metabolites of cyclobrassinin biotransformation, the process was scaled up as described in the experimental section. Time-course experiments established that no extractable metabolites were present in the acidic, basic and mycelia extracts. In scaled up experiments therefore only neutral broth extractions were carried out.

2.2.2.1 Metabolism of cyclobrassinin by Phoma lingam virulent isolates

The time-course experiments established that cyclobrassinin (2) is biotransformed by P. lingam isolate BJ-125 to yield mainly a single metabolite, R, 0.30 [CH2Cl2-MeOH (95 : 5)] with retention time 7.5 minutes in the HPLC chromatogram of the neutral ether extract. In a series of scale up experiments this metabolite was isolated and characterized. At the end of the biotransformation period (24-30 hours) fungal cultures were filtered and the broth extracted with an equivalent amount of diethyl ether. The ether extract was processed and analyzed by TLC and HPLC as described in the experimental section. The
metabolite was isolated by preparative TLC and characterized by spectroscopic techniques. It was identified as dioxibrassinin (36) (Scheme 40), a known cruciferous phytoalexin, first isolated from cabbage (*B. oleracea*) (Monde et al., 1991a). The isolated amount of 36 accounted for ca. 30-40% of 2 added to the cultures after a 1 day incubation period. The time-course experiments also showed that dioxibrassinin is metabolized further by the fungus. The chromatograms of samples taken after 96 hours showed no peaks attributable to products of phytoalexin metabolism by the fungus. Dioxibrassinin is degraded further to undetermined products.

**Scheme 40**

\[ 
\begin{align*}
\text{N} & \quad \text{SCH}_3 \\
\text{H} & \\
\text{N} & \quad \text{SCH}_3 \\
\text{H} & \\
\end{align*}
\]

\[ 
\begin{align*}
\text{H} & \quad \text{N} & \quad \text{SCH}_3 \\
\text{H} & \\
\end{align*}
\]

*Pathway for the biotransformation of cyclobrassinin (2) by virulent *P. lingam*

---

2.2.2.2 *Metabolism of cyclobrassinin by Phoma lingam avirulent isolate*  

*Unity*  

Time-course experiments established that cyclobrassinin is metabolized by avirulent *P. lingam* isolate Unity to yield two products, *R*₁ 0.40, 0.20 [RP-8 plate, CH₃CN-H₂O (40 : 60)] with retention times of 7.2 and 12.2 minutes, respectively, in the HPLC
chromatograms of the neutral ether extract. In a series of scale up experiments these biotransformation products were isolated in sufficient quantities for structure elucidation and identification. After incubation for a time period (12-28 hours) the fungal cultures were filtered and the broth extracted with an equivalent amount of diethyl ether. The ethereal extract was processed and analyzed by TLC and HPLC as described in the experimental section. Flash column chromatography or preparative TLC resulted in the isolation of the two metabolites. Typically the amount of isolated metabolites was ca. 40-50% of the original amount of 2 after 1 day incubation period. Characterization by spectroscopic techniques led to their respective identification as the novel 3-methylenaminoindole-2-thione (34) and brassilexin (29) a known cruciferous phytoalexin, first isolated from *B. juncea* (Devys et al., 1988). These compounds were further metabolized by the fungus; however no further products of metabolism were detected. The HPLC chromatograms of samples taken after 96 hours from cultures incubated with cyclobrassinin showed no peaks assignable to phytoalexin metabolites, that is the samples were identical to fungal control cultures. It is likely that 3-methylenaminoindole-2-thione and brassilexin are metabolized further to give polar undetected products. In order to establish the sequence of the biotransformation steps and hence the biotransformation pathway each of the two metabolites was subjected to further incubation with the fungus. Brassilexin (29) was biotransformed by the fungus but no further metabolites were detected or isolated; 3-methylenaminoindole-2-thione (34) was rapidly (within 24 hours) biotransformed to yield brassilexin. The latter was then metabolized further to undetermined products. 3-Methylenaminoindole-2-thione was found to be a relatively unstable metabolite and readily oxidized to brassilexin during separation on normal phase silica gel TLC plates. Under the conditions employed for the HPLC analysis the chromatograms of this compound indicated partial conversion to brassilexin, as shown by two peaks with retention times 7.2 (major) and 12.2 (minor) minutes. On a semi-preparative HPLC scale the peaks at retention times 7.2 and 12.2
minutes were separately collected. The samples were concentrated and analyzed by HPLC. Whereas the sample collected at 12.2 minutes showed only one peak at the same retention time, the sample collected at 7.2 minutes showed two peaks with retention times 7.2 and 12.2 minutes.

Further incubations of 3-methylenaminoindole-2-thione with avirulent *P. lingam* isolate Unity were monitored by TLC [RP-8 plates, CH$_3$CN-H$_2$O (40 : 60)]. The TLC plates of samples taken after 0, 2, 3 hours showed only a single spot (R$_f$ 0.40) corresponding to 3-methylenaminoindole-2-thione. Those of samples taken after 5, 6, 8 hours showed two spots (R$_f$ 0.40 and 0.20) corresponding to 3-methylenaminoindole-2-thione and brassilexin. TLC plates of samples taken after 12 hours showed mainly a single spot corresponding to brassilexin. After 24 hours no spot corresponding to 3-methylenaminoindole-2-thione or brassilexin were detected by TLC. Similar TLC analyses of the 3-methylenaminoindole-2-thione control samples showed that a small amount of the compound (ca. 10%) was converted to brassilexin (after 24 hours) under these incubation conditions. These results established that 3-methylenaminoindole-2-thione is an intermediate to brassilexin during fungal transformation. From the results of biotransformation of cyclobassinin and metabolites by *P. lingam* avirulent isolates the major pathway shown in Scheme 41 is proposed.

### 2.2.2.3 Metabolism of cyclobassinin by *Rhizoctonia solani* virulent isolate AG2-1

As shown by time-course experiments, cyclobassinin (2) was metabolized by *R. solani* virulent isolate AG2-1 to yield three main products. Scale up experiments were conducted and the three metabolites isolated by flash column chromatography and / or preparative TLC and characterized by spectroscopic techniques. They were identified as 2-mercaptoindole-3-carboxaldehyde (32), the cruciferous phytoalexin brassicanal A (52), and 5-hydroxybrassicanal A (129). Brassicanal A is a phytoalexin first isolated from
Chinese cabbage (Monde et al., 1990). The $R_f$ values and HPLC retention times in minutes are summarized in Table 2.7. Isolated metabolites amounted to ca. 50-60% of 2 added to the fungal cultures after 4 days incubation. In order to establish the sequence of the biotransformation steps and to map out the pathway(s), each of the metabolites 2-mercaptoindole-3-carboxaldehyde (32), brassicanal A (52), and 5-hydroxybrassicanal A (129) was subjected to further biotransformation by virulent *R. solani* isolate AG2-1.

Scheme 41 $^a$

$^a$ Pathway for biotransformation of cyclo brassinin by avirulent *P. lingam*. 

85
2-Mercaptoindole-3-carboxaldehyde (32) yielded brassicanal A, brassicanal A sulfoxide (106), and 5-hydroxybrassicanal A. Brassicanal A (52) gave brassicanal A sulfoxide, and 5-hydroxybrassicanal A. Although 5-hydroxybrassicanal A (131) was metabolized further by the fungus, no biotransformation products were detected or isolated. This compound is likely metabolized to undetermined polar products. The chromatograms of samples taken after 7 days showed no peaks attributable to cyclobrassinin and its metabolites. From the results of the biotransformation of cyclobrassinin by *R. solani* virulent isolate AG2-1, the pathway for biotransformation shown in Scheme 42 is proposed.

**Table 2.7** HPLC peak retention times and R<sub>f</sub> values for cyclobrassinin (2) and products of biotransformation by *R. solani*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;f&lt;/sub&gt; value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HPLC peak retention time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclobrassinin (2)</td>
<td>0.80</td>
<td>24.3</td>
</tr>
<tr>
<td>2-Mercaptoindole-3-carboxaldehyde (32)</td>
<td>0.37</td>
<td>18.8</td>
</tr>
<tr>
<td>Brassicanal A (52)</td>
<td>0.51</td>
<td>10.7</td>
</tr>
<tr>
<td>5-Hydroxybrassicanal A (129)</td>
<td>0.26</td>
<td>5.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> R<sub>f</sub> values determined in CH<sub>2</sub>Cl<sub>2</sub>-MeOH (95 : 5).
2.2.3 Characterization and identity of cyclobrassinin metabolites

Dioxibrassinin (36) was found to be optically active ([α]_D = +21.3, c 0.0024, MeOH). The molecular formula was determined to be C_{11}H_{12}N_2O_2S_2 on the basis of HREIMS (m/z found 268.0345 M^+, calcd. 268.0340) in combination with ^1H and ^13C NMR spectral data. The molecular formula formally represents the addition of a hydrogen peroxide (H_2O_2) unit to cyclobrassinin. The FTIR spectrum exhibited characteristic bands at 3273, 3223 (OH, NH) and 1720 cm\(^{-1}\) (C=O). The ^1H NMR spectrum showed signals indicating the presence of four adjacent aromatic protons at δ 7.36 (d, J = 7.5 Hz, 1H), 7.27 (dd, J = 7.5, 7.5 Hz, 1H), 7.01 (dd, J = 7.5, 7.5 Hz, 1H), 6.89 (d, J = 7.5 Hz,
1H) and two geminal protons at $\delta$ 4.32 (d, $J = 14$ Hz, 1H) and 3.98 (d, $J = 14$ Hz, 1H). The $^{13}$C NMR spectrum clearly showed the signals of 11 carbons: one thiocarbonyl carbon at $\delta$ 201.9 (s), one carbonyl carbon at $\delta$ 180.6 (s), six aromatic carbons at $\delta$ 142.7 (s), 131.1 (2C, s, d), 125.9 (d), 123.9 (d), 111.4 (d), one quaternary carbon at $\delta$ 76.3 (s), one methylene carbon at $\delta$ 53.3 (t), and one thiomethyl carbon at $\delta$ 18.2 (q). Compared to cyclobrassinin this compound had one less degree of unsaturation. The methylene protons are diastereotopic indicating that they are adjacent to a stereogenic center. Unlike cyclobrassinin, the signals for a thiocarbonyl, a carbonyl and a quaternary carbon were observed for this compound. The downfield shift of the quaternary carbon signal at $\delta$ 76.3 (s) indicated that it was attached to oxygen. These structural features suggested that this compound could be derived from cyclobrassinin by oxidative ring opening. On the basis of the spectroscopic data and comparison with an authentic sample, this compound was identified as the cruciferous phytoalexin dioxibrassinin (36). The data was consistent with that reported (Monde et al., 1991) for the compound except for the specific rotation. The reported value for the specific rotation ($[\alpha]_D = -7.6$) has the opposite sign as well as a lower absolute value. As shown in Scheme 43 (Pedras and Okanga, 1998c), enzymatic dioxygenation of the double bond at the 2,3-position could explain the formation of dioxibrassinin from cyclobrassinin via intermediate 130.
The $^1$H NMR spectrum (CD$_2$Cl$_2$) of 3-methylenaminooindole-2-thione (34) showed signals for three D$_2$O exchangeable protons at $\delta$ 11.13 (br s, 1H), 8.98 (br s, 1H) and 6.25 (br s, 1H). In addition there were signals for four coupled aromatic protons characteristic of a 2,3-disubstituted indole nucleus and a double doublet at $\delta$ 8.15. Decoupling experiments established that the signal at $\delta$ 8.15 was coupled to the signals at $\delta$ 6.25 and 11.13. Irradiation of the signal at $\delta$ 6.25 collapsed the double doublet at $\delta$ 8.15 to a doublet with a coupling constant of 15 Hz. Similarly irradiation of the signal at $\delta$ 11.13 reduced the double doublet at $\delta$ 8.15 to a doublet with a coupling constant of 8 Hz. The $^{13}$C NMR spectrum in CD$_2$Cl$_2$ showed signals for nine carbons: a thiocarbonyl carbon at $\delta$ 179.0 (s), eight sp2 carbons; three quaternary and five CH. In CD$_3$CN the $^{13}$C NMR spectrum showed signals for 11 carbons while in CD$_3$OD the spectrum showed signals for 22 carbons. This was an indication that the compound is capable of existing in various tautomeric forms depending on the solvent. The HREIMS ($m / z$ found 176.0412 M$^+$,
calcd. 176.0408) gave a molecular formula C₉H₈N₂S. The FTIR spectrum showed characteristic absorption bands at 3238 (NH) and 1641 cm⁻¹ (aromatic C=C). Compared to cyclobrassinin this compound had one less degree of unsaturation and lacked signals for thiomethyl and methylene protons. Unlike cyclobrassinin this compound showed a signal at δ 179.0 (s) for a thiocarbonyl carbon, an indication that the compound derived from cyclobrassinin by modification of ring C. On the basis of the spectroscopic data this compound was characterized and identified as 3-methylenaminoindole-2-thione (34). Depending on the solvent this compound may exist also in the tautomeric forms 3-methylenaminoindole-2-thiol (127), and 3-(methylimino)indole-2-thiol (128) (Scheme 41). Initially, to confirm the structure of thione 34 acetylation and hydrolysis were considered. Thus treatment of 3-methylenaminoindole-2-thione with excess acetic anhydride and pyridine at room temperature yielded both the mono- and diacetyl derivatives 131 and 132 (Scheme 44) respectively.

Scheme 44

![Scheme 44 Diagram]

[a] Reagents: (a) Ac₂O, py.
The latter compound being the major product. Acid hydrolysis of 34 with CF₃COOH / H₂O yielded 2-mercaptopindole-3-carboxaldehyde (32) as the main product with traces of brassilexin (29) (Scheme 45).

\[
\text{Scheme 45}^a
\]

\[
\begin{align*}
34 & \xrightarrow{a} \quad 32 + 29 \\
\text{[structure diagram]}
\end{align*}
\]

\[^a\text{Reagents: (a) CF₃COOH, H₂O.}\]

As a final proof of structural assignment for thione 34 attention was turned to its synthesis. Two apparent methods were (a) treatment with ammonia and (b) reduction of amino derivatives of 3-hydroxymethylene-2-indolinethione (or equivalent tautomer, e.g. 32) similar to the preparation of 3-hydroxymethyleneoxindole (134) derivatives (Wenkert et al., 1956, 1959). Attempted synthesis of 3-methylenaminoindole-2-thione (34) via 3-chloromethylene-2-indolinethione (133) (Scheme 46) was unsuccessful. This method was employed previously for the preparation of the corresponding 3-aminomethyleneoxindole (136) from 134 via 135 (Scheme 47) (Behringer and Weissauer, 1952). Unlike 3-hydroxymethyleneoxindole (134), treatment of 2-mercaptopindole-3-carboxaldehyde (32) with SOCl₂ led to unidentified decomposition products. Finally, synthesis of 34 from 2-mercaptopindole-3-carboxaldehyde (32) via the
oxime (33) (Scheme 8 in section 1.4.1.1) (Pedras and Okanga, 1998a) was accomplished. Thus, oximation of 32 under standard conditions (Wenkert et al., 1958) yielded quantitatively oxime 33, which was readily reduced to the desired thione (34) with NaBH₃CN in the presence of TiCl₃ (Leeds and Kirst, 1988).

Scheme 46 *

\[ \text{CHO} \quad \overset{a}{\longrightarrow} \quad \text{Cl} \]

32 \quad \text{133}

\[ \overset{b}{\searrow} \]

34

* Reagents: (a) SOCl₂; (b) NH₃ / MeOH
Brassilexin (29) had a molecular formula C_{6}H_{6}N_{2}S as shown by HREIMS (m/z found 174.0253 M^+, calcd. 174.0252). The ^1H NMR spectrum showed signals for five aromatic protons. Four of these were coupled and characteristic of a 2,3-disubstituted indole nucleus. The spectrum further showed the presence of a D_{2}O exchangeable signal at δ 9.81 (br s, 1H). This compound had the same degree of unsaturation as cyclobrazasin indicating that it was also tricyclic. However, it lacked the signals attributable to a methylene and a thiomethyl. The ^13C NMR spectrum showed nine signals for nine aromatic carbons: four quaternary and five CH. The FTIR spectrum showed characteristic absorption bands at 3274 (NH), 3140, 2924, 2853, isothiazole substructure, confirmed by sharp bands at 856 and 744 cm⁻¹. On the basis of the spectroscopic data, this
compound was identified as the cruciferous phytoalexin brassilexin (29). Identity was further confirmed by comparison of the physical data with those of an authentic sample obtained by synthesis (Devys and Barbier, 1993; Pedras and Okanga, 1998a). The synthesis of brassilexin from 3-methylenaminoindole-2-thione (34) (Scheme 8 in section 1.4.1.1) closely mimicked the fungal detoxification pathway of the cruciferous phytoalexin cyclobrassinin (2) by P. lingam isolate Unity (Scheme 41). This synthetic route afforded the best overall yield of brassilexin to date while following the simplest process in terms of purification and reaction conditions (Pedras and Okanga, 1998a).

2-Mercaptoindole-3-carboxaldehyde (32) was found to have the molecular formula C₉H₇NOS on the basis of HREIMS (m/z found 177.0247 M⁺, calcd. 177.0248) in combination with ¹H and ¹³C NMR spectral data. The ¹H NMR spectrum showed signals for four aromatic protons characteristic of a 2,3-disubstituted indole ring. In addition, the spectrum showed signals for an aldehydic proton at δ 9.65 (s, 1H) and a D₂O exchangeable proton at δ 10.58 (br s, 1H). The ¹³C NMR spectrum showed nine signals corresponding to nine carbon atoms: a carbonyl carbon at δ 187.1 (s), eight sp² carbons; four quaternary and four methine. Compared to cyclobrassinin, this compound also lacked the signals attributable to thiomethyl and methylene groups. The signal due to a carbonyl group is newly observed for this compound. The compound also has one less degree of unsaturation. These differences were an indication that the compound derived from cyclobrassinin by degradation of the C-ring. The FTIR spectrum showed characteristic absorption bands at 3229 (NH) and 1621 cm⁻¹ (C=O). On the basis of the spectroscopic data this compound was identified as 2-mercaptoindole-3-carboxaldehyde (32). To further confirm identity of aldehyde 32 synthesis was considered. 2-Mercaptoindole-3-carboxaldehyde (32) could be synthesized from oxindole by (a) thiation followed by formylation or (b) formylation followed by thiation. Synthesis following the former method was first attempted. Formylation of oxindole with HCOOEt / NaOEt (Wenkert et al., 1956) yielded 3-hydroxymethylene-2-indolmethione (134). Thiation (Hino et al.,
1969) of 134 (Scheme 48) was, however, unsuccessful. Starting material was recovered together with some elemental sulfur. The elemental sulfur was most likely a product of decomposition of $P_2S_5$. In another attempt protection of 3-hydroxymethylene-2-indolinethione (134) as the acetal was considered. Refluxing a solution of 134 in NH$_4$Cl / MeOH (De Graw et al., 1961) provided enol ether 137 and not the acetal. All attempts to convert 137 to thione 138 (Scheme 48) failed. Finally, 2-mercaptoindole-3-carboxaldehyde (32) was synthesized by thiation of oxindole to 2-indolinethione (31) followed by formylation with NaH / HCOOEt as outlined in Scheme 8 (section 1.4.1.1) (Okanga and Pedras, 1998a). Formylation of thione 31 with NaOEt / HCOOEt as for oxindole was also unsuccessful.

Scheme 48 $^a$

$^a$ Reagents and conditions: (a) $P_2S_5$, benzene, reflux; (b) NH$_4$Cl, MeOH, reflux.
Brassicanal A (52) was determined to have the molecular formula C_{10}H_{10}NOS on the basis of HREIMS (m/z found 191.0402 M^+, calcd. 191.0405) in combination with ^1H and ^13C NMR spectral data. The ^1H NMR spectrum showed signals for four aromatic protons characteristic of a 2,3-disubstituted indole nucleus, an aldehydic proton at δ 10.15 (s, 1H), a thiomethyl at δ 2.64 (s, 3H) and a D₂O exchangeable proton also at δ 10.15 (br s, 1H). The ^13C NMR spectrum showed signals for ten carbons: a carbonyl carbon at δ 184.7 (s), eight sp2 carbons: four quaternary and four methine, and one sp3 carbon for a thiomethyl at δ 16.9 (q). In comparison with cyclobassinin, the major differences were the presence of a carbonyl group and the absence of a methylene group. These differences and the fact that this compound had one less degree of unsaturation also indicated that the compound is derived from cyclobassinin by modification of ring C. The FTIR spectrum showed characteristic absorption bands at 3152 (NH), and 1627 cm⁻¹ (C=O). On the basis of the spectroscopic data the compound was identified as the brassica phytoalexin brassicanal A (52). Identity was further confirmed by comparison of the physical data (TLC, NMR, FTIR) with those of an authentic sample obtained by synthesis (Pedras and Khan, 1996; Pedras and Okanga, 1998a). The synthesis of brassicanal A from 2-mercaptopindole-3-carboxaldehyde (32) (Scheme 13 in section 1.4.1.1) closely mimicked the fungal detoxification pathway of the cruciferous phytoalexin cyclobassinin (2) by R. solani isolate AG2-1 (Scheme 42). This synthesis of brassicanal A is a great improvement to the previously reported procedure, which employed methylation of indolinethione (31) followed by Vilsmeier formylation of the corresponding 2-thiomethyl ether (51) (Scheme 13) (Monde et al., 1990; Pedras and Khan, 1996).

5-Hydroxybrassicanal A (129) had a molecular formula C_{10}H_{10}NO₂S on the basis of HREIMS (m/z found 207.0353 M^+, calcd. 207.0354) in combination with ^1H and ^13C NMR spectral data. The FTIR spectrum showed characteristic absorption bands at 3171 (NH, OH) and 1612 cm⁻¹ (C=O). The ^1H NMR spectrum showed the following significant signals: aromatic proton signals of a 1, 2, 4-trisubstituted benzene at δ 7.48 (d,
J = 2.5 Hz, 1H) 7.21 (d, J = 9 Hz, 1H) 6.74 (dd, J = 9, 2.5 Hz, 1H), thiomethyl protons signal at δ 2.64 (s, 3H), a singlet signal at δ 10.07 attributable to an aldehyde proton, and a broad D$_2$O exchangeable proton signal at δ 9.96 (s). These spectral data in combination with $^{13}$C NMR, and HMQC spectral data suggested the presence of either a 2,3,5- or a 2,3,6-trisubstituted indole moiety. The $^1$H and $^{13}$C NMR spectral data also suggested the existence of a formyl group (δ$_H$ 10.07; δ$_C$ 185.7), and its upfield-shifted $^{13}$C chemical shift indicated that the formyl group could be attached at an aromatic ring. Taking into account the molecular formula (C$_{10}$H$_6$NO$_2$S), the other substituent should be a hydroxyl group. The $^{13}$C chemical shift of δ 154.9 (C-5 or C-6) indicated that this carbon was hydroxylated. These spectral data indicated that this compound was an indole compound having thiomethyl, formyl and hydroxyl groups as substituents. Except for the hydroxylation of the benzene ring, the other structural features of this compound were similar to those of brassicanal A. The positions of the substituents were determined by HMBC experiments in which the following long-range C-H correlations were observed: H-4 / C-6, H-4 / C-7a, H-6 / C-4, H-6 / C-7a, H-7 / C-5, H-7 / C-3a, H-1’ / C-3a and SCH$_3$ / C-2. The position of the hydroxyl group was determined to be either at C-5 or C-6. Comparison of the spectroscopic data of 129 with that of 5-hydroxycamalexin (109) led to assignment of the structure as 5-hydroxybrassicanal A. It is worth noting that the fungus R. solani was shown to detoxify camalexin via 5-hydroxycamalexin (Scheme 32) (Pedras and Khan, 1997).

2.2.4 Antifungal activity of cyclobrassinin and metabolites

2.2.4.1 Bioassays with Phoma lingam isolates

The antifungal activity of cyclobrassinin and each of its metabolites against virulent and avirulent P. lingam isolates was tested by determining the inhibition of spore germination and radial mycelial growth. The compounds were bioassayed at two
concentrations of $1 \times 10^{-4}$ M and $5 \times 10^{-4}$ M against *P. lingam* virulent and avirulent isolates BJ-125 and Unity, respectively. The results of the bioassays are presented in Tables 2.8 and 2.9. Dioxibrassinin, the metabolite of cyclobrassinin biotransformation by the virulent isolate BJ-125 is less inhibitory to spore germination and radial mycelial growth. This metabolite is transformed further to undetermined less toxic polar products as shown by the density of mycelial growth in comparison with the control cultures. This clearly is a detoxification process. The results of the bioassays with *P. lingam* avirulent isolate Unity show that the metabolites brassilexin and 3-methyleneaminindoledo-2-thione are much more toxic to the fungus than cyclobrassinin. At the two concentrations of $1 \times 10^{-4}$ M and $5 \times 10^{-4}$ M the two metabolites completely inhibited spore germination and mycelial growth over the seven-day period. Since 3-methyleneaminindoledo-2-thione is readily converted to brassilexin it was difficult to establish if the essays were indeed representative of the compound. It is possible that 3-methyleneaminindoledo-2-thione could be converted to brassilexin under the bioassay conditions. The two metabolites 3-methyleneaminindoledo-2-thione and brassilexin were transformed further by the fungus to less toxic products. This is supported by the observation that there was some inhibition of mycelial growth in cultures incubated with cyclobrassinin over the period (0-24 h) the compound and its metabolites were present in the cultures. After complete metabolism of cyclobrassinin and metabolites the mycelial density in the liquid cultures increases rapidly. After four days (96 h) the mycelial mass of cultures incubated with cyclobrassinin was similar to that of control cultures. Overall the biotransformation of cyclobrassinin by avirulent isolate Unity is also a detoxification process. These results indicated that cyclobrassinin (2) is detoxified via the phytoalexins brassilexin (29) or dioxibrassinin (36), depending on the particular fungal isolate, as shown in Schemes 40, 41.
Table 2.8 Bioassay of cyclobrazin (2) and metabolites against virulent *P. lingam* isolate BJ-125 after 72 hours incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>% Inhibition a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclobrazin (2)</td>
<td>$1 \times 10^{-4} \text{ M}$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4} \text{ M}$</td>
<td>21</td>
</tr>
<tr>
<td>Dioxibrazin (36)</td>
<td>$1 \times 10^{-4} \text{ M}$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4} \text{ M}$</td>
<td>0</td>
</tr>
</tbody>
</table>

a % inhibition = 100 - [(growth in treated / growth in control) × 100]; results are the mean of at least three separate experiments, standard deviation ± 0.5

Table 2.9. Bioassay of cyclobrazin (2) and metabolites against avirulent *P. lingam* isolate Unity after 72 hours incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>% Inhibition a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclobrazin (2)</td>
<td>$1 \times 10^{-4} \text{ M}$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4} \text{ M}$</td>
<td>7</td>
</tr>
<tr>
<td>Brassilexin (29)</td>
<td>$1 \times 10^{-4} \text{ M}$</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4} \text{ M}$</td>
<td>100</td>
</tr>
<tr>
<td>3-methylenaminoindole-2-thione (34)</td>
<td>$1 \times 10^{-4} \text{ M}$</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4} \text{ M}$</td>
<td>100</td>
</tr>
</tbody>
</table>

a % inhibition = 100 - [(growth in treated / growth in control) × 100]; results are the mean of at least three separate experiments, standard deviation ± 0.5

2.2.4.2 Bioassays with *Rhizoctonia solani*

The antifungal activity of cyclobrazin and each of its metabolites with *R. solani* was tested by determining the inhibition of radial mycelial growth. The compounds were
bioassayed at two concentrations of $1 \times 10^{-4}$ M and $5 \times 10^{-4}$ M against mycelia of *R. solani* virulent isolate AG2-1. The results of the bioassays are presented in Table 2.10. These results show that cyclobraassinin is more toxic to the fungus than any of its metabolites. The toxicity of the compounds decreases in the order cyclobraassinin, 2-mercaptoindole-3-carboxaldehyde, brassicanal A, 5-hydroxybrassicanal A. This is a detoxification process. These results also showed that the fungal pathogen *R. solani* AG2-1 detoxifies the phytoalexin cyclobraassinin (2) via another cruciferous phytoalexin, brassicanal A (52).

**Table 2.10** Bioassay of cyclobraassinin (2) and metabolites against virulent *R. solani* isolate AG2-1 after 72 hours incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>% Inhibition a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclobraassinin (2)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>27</td>
</tr>
<tr>
<td>2-Mercaptoindole-3-carboxaldehyde (32)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>23</td>
</tr>
<tr>
<td>Brassicanal A (52)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>14</td>
</tr>
<tr>
<td>5-Hydroxybrassicanal A (129)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>5</td>
</tr>
</tbody>
</table>

a $\%$ inhibition = 100 - [(growth in treated / growth in control) $\times$ 100]; results are the mean of at least three separate experiments, standard deviation $\pm$ 0.5

### 2.2.5 Effect of cyclobraassinin on phytotoxin production

As discussed in section 2.1.5 the phytoalexin brassinin and its homolog, methyl tryptamine dithiocarbamate have been observed to inhibit the production of nonselective
phytotoxins in liquid cultures of virulent *P. lingam* isolates. The virulent isolate BJ-125 was employed in biotransformations studies with cyclobassinin. Since cyclobassinin was rapidly metabolized (within 24 hours) by the fungus, its effect on the production of nonselective phytotoxins (detected in cultures after 48-96 hours) was not apparent. Likewise its effect on the host selective phytotoxin phoralide was not observed as cyclobassinin was added to 48 hour-old cultures when the fungus was already producing the toxin.

### 2.3 Other phytoalexin analogues

This section describes and discusses the results of metabolism of the phytoalexin analogues methyl tryptamine carbamate (4), N$_6$-carbomethoxyindole-3-methanamine (5) and cyclobassinin homologue (6). These analogues were chosen in order to probe the effect of structural modifications on fungal metabolism in comparison with phytoalexins. In particular the effects of ring size and substitution of oxygen for sulfur were investigated.

#### 2.3.1 Synthesis and characterization

##### 2.3.1.1 Synthesis and characterization of methyl tryptamine carbamate

Methyl tryptamine carbamate (4) was synthesized from tryptamine (112) as outlined in Scheme 49 and described in the experimental section. In a one-pot procedure tryptamine was treated with N$_{2}$O-bis(trimethylsilyl)acetamide followed by addition of methyl chloroformate to yield the carbamate (67%). This is a mild procedure for the conversion of amines to carbamates (Raucher and Jones, 1985). The transformation involves initial conversion of the amine to the corresponding trimethylsilylamine followed by cleavage of the silicon-nitrogen bond by the chloroformate. The molecular formula was determined to be C$_{12}$H$_{14}$N$_{2}$O$_{2}$ on the basis of HREIMS ($m/z$ found 218.1054 M$^+$, calcd. 218.1055) in combination with $^1$H and $^{13}$C NMR data. The $^1$H and $^{13}$C NMR spectra clearly showed
Scheme 49

\[ \text{112} \xrightarrow{a, b} \text{4} \]

*Reagents: (a) N,O-bis(trimethylsilyl)acetamide, CH\(_2\)Cl\(_2\); (b) methyl chloroformate.*

Scheme 50

\[ \text{4} \xrightarrow{a} \text{139} \]

\[ \text{140} \]

*Reagents: (a) pyridinium bromide perbromide; DBU.*
signals for a methoxy group at $\delta_H$ 3.67 (s, 3H), $\delta_C$ 52.0 (q), two methylene groups at $\delta_H$ 3.53 (m, 2H) and 2.98 (m, 2H), $\delta_C$ 41.4 (t) and 25.8 (t) and a C-3 substituted indole nucleus. The FTIR showed characteristic absorption bands at 3409, 3322 (NH) and 1700 cm$^{-1}$ (C=O).

Attempted cyclization (Scheme 50) of methyl tryptamine carbamate (4) to give a cyclobraasin analogue 139 was unsuccessful, methyl 2-oxotryptamine carbamate (140) was formed instead.

2.3.1.2 Synthesis and characterization of $N_b$-carbomethoxyindole-3-methanamine

$N_b$-carbomethoxyindole-3-methanamine (5) was synthesized from indole-3-methanamine (21) following the same procedure as for methyl tryptamine carbamate (section 2.3.1.1). The molecular formula was determined to be C$_{11}$H$_{12}$N$_2$O$_2$ on the basis of HREIMS ($m/z$ found 204.0895, calcd. 204.0899) in combination with $^1$H and $^{13}$C NMR data. The $^1$H and $^{13}$C NMR spectra showed the signals for a methoxy group at $\delta_H$ 3.70 (s, 3H); $\delta_C$ 52.2 (q), one methylene group at $\delta_H$ 4.56 (d, $J = 5$ Hz, 2H); $\delta_C$ 36.8 (t) and a C-3 substituted indole nucleus. The FTIR showed characteristic absorption bands at 3407, 3322 (NH) and 1699 cm$^{-1}$ (C=O).

Attempted cyclization of this compound (Scheme 51) to a cyclobraasin analogue 141 was unsuccessful. $N_b$-carbomethoxyindole-3-methanamine decomposed to unidentified products under the reaction conditions.
2.3.1.3 Synthesis and characterization of cyclobrassinin homologue

Cyclobrassinin homologue (6) was synthesized from methyl tryptamine dithiocarbamate (3) in the same manner as for the synthesis of cyclobrassinin from brassinin (section 2.2.1). Thus methyl tryptamine dithiocarbamate was brominated with pyridinium bromide perbromide followed by dehydrobromination with DBU to give homologue 6. This process also yielded a small amount of compound 142 (cyclobrassinin homologue metabolite) as a side product (see sections 2.3.2.3 and 2.3.3.2).

2.3.2 Biotransformation Studies

Time-course and scaled up biotransformation experiments were conducted as discussed for methyl tryptamine dithiocarbamate and cyclobrassinin (sections 2.1.2, 2.2.2).
2.3.2.1 Metabolism of methyl tryptamine carbamate by Phoma lingam isolates

Time-course experiments showed that methyl tryptamine carbamate (4) underwent little (<10%) transformation when incubated with *P. lingam* virulent isolate BJ-125. After 14 days incubation the major peak in the HPLC chromatogram of the neutral extract was that due to starting material (methyl tryptamine carbamate). The only other significant peaks were those corresponding to phytotoxins produced by the fungus. The acidic extract showed a broad peak at retention time 4.9 minutes in addition to those of recovered starting material and the phytotoxins. In a scaled up procedure the metabolite with retention time 4.9 minutes was isolated, characterized and identified as indole-3-acetic acid (117).

Time-course experiments showed that methyl tryptamine carbamate (4) is biotransformed by *P. lingam* avirulent isolate Unity to yield two metabolites with retention times 5.0 and 6.9 minutes in the HPLC chromatograms of the neutral ether extract. By scaling up the biotransformation process the two metabolites were isolated, characterized by spectroscopic techniques and identified as tryptophol (121) and indole-3-carboxylic acid (104). Isolated metabolites accounted for ca. 40-50% of the initial amount of 4 after 14 days incubation. Their *R*ₚ and HPLC peak retention times are shown in Table 2.1.

2.3.2.2 Metabolism of *N₅*-carbomethoxyindole-3-methanamine by Phoma lingam isolates

Time-course experiments with virulent (BJ-125) *P. lingam* isolates showed that *N₅*-carbomethoxyindole-3-methanamine (5) was biotransformed by the fungus. However, no metabolites were detected or isolated. Similar experiments with avirulent (Unity) isolates indicated no biotransformation of *N₅*-carbomethoxyindole-3-methanamine by this fungal isolate. The compound was stable in the cultures with *P. lingam* avirulent isolate Unity for up to 14 days incubation. Biotransformation studies of *N₅*-carbomethoxyindole-3-
methanamine by both virulent and avirulent *P. lingam* isolates were therefore not pursued further.

**2.3.2.3 Metabolism of cyclobrassinin homologue by *Phoma lingam* isolates**

As shown by time-course experiments, the biotransformation of cyclobrassinin homologue (6) by either virulent (BJ-125) or avirulent (Unity) isolates of *P. lingam* resulted in the identification of one metabolite with retention time 18 minutes (broad peak with variable retention time). In a series of scale up experiments this metabolite was isolated and characterized by spectroscopic techniques. The amount of isolated metabolite was ca. 50% of the initial amount of 6 after 1 day incubation period. Structure (142) (Figure 2.1) is proposed for this compound.

![Structure of cyclobrassinin homologue metabolite (142).](image)

**Figure 2.1** Structure of cyclobrassinin homologue metabolite (142).

**2.3.3 Characterization and identity of metabolites**

**2.3.3.1 Methyl tryptamine carbamate metabolites**

Indole-3-carboxylic acid (104), indole-3-acetic acid (117) and Tryptophol (121) were identified from the physical data, as described in section 2.1.3, and comparison with authentic samples.
2.3.3.2 *Cyclobraisin* homologue *metabolite*

The $^1$H NMR spectrum of metabolite 142 showed signals for a thiomethyl group at $\delta$ 2.41 (s, 3H), two methylene groups at $\delta$ 4.31-4.48 (m, 1H); 4.22-4.31 (m, 1H) and 2.81-2.90 (m, 1H); 2.44-2.56 (m, 1H), four coupled aromatic protons characteristic of a 2,3-disubstituted indole ring, and a signal for a D$_2$O exchangeable proton at $\delta$ 10.79 (br s, 1H). The methylene protons were nonmagnetically equivalent as shown by their complex coupling patterns. The HREIMS ($m / z$ found 248.0436, calcd. 248.0442) gave an apparent molecular formula C$_{12}$H$_{12}$N$_2$S$_2$, which corresponded to that of the parent compound 6. The FTIR displayed characteristic absorption bands at 3144 (NH), 1587, 1468, and 1433 cm$^{-1}$ (C=O). The $^{13}$C NMR showed 12 signals for six aromatic carbons at $\delta$ 143.1 (s), 135.0 (s), 129.5 (d), 124.9 (d), 124.1 (d) and 110.6 (d), a thiocarbonyl carbon at $\delta$ 206.6 (s), a low field sp$^2$ quaternary carbon at $\delta$ 173.0 (s) and four aliphatic carbons at $\delta$ 77.1 (s), 60.9 (t), 41.3 (t) and 14.4 (q). The low field signal at $\delta$ 173.0 appeared unlikely to be due to a carbonyl group as the FTIR showed no signal assignable to a C=O group. On the other hand, the low field chemical shift ($\delta$ 77.1) of the quaternary aliphatic carbon suggested attachment to oxygen. Also the chemical shifts of the methylene carbons ($\delta$ 60.9, 41.3) were significantly downfield compared to cyclobraassinin homologue 6 ($\delta$ 51.9, 23.9). The signal for the thiomethyl carbon ($\delta$ 14.4) in this compound was shifted upfield compared to cyclobraassinin homologue ($\delta$ 16.4). The $^{13}$C NMR data suggested the presence of oxygen in this compound. Since the oxygen was not in the form of an alcohol or a carbonyl it must be in the form of an ether. The MS data showed no evidence for the presence of oxygen, but the ion at $m / z$ 248 could be a fragment ion obtained by loss of oxygen. The molecular formula of the compound is most likely C$_{12}$H$_{12}$N$_2$OS$_2$. The base peak in the EIMS at $m / z$ 175 corresponded to the fragment ion (C$_{10}$H$_{8}$NS)$^+$, which could be obtained by the consecutive loss of oxygen and a C$_2$H$_3$NS unit, that is M$^+$ - C$_2$H$_3$NOS. The absence of oxygen-containing fragment ions in
the EIMS and the lack of a C=O signal in the FTIR spectrum ruled out a 2-oxo-type structure in favor of a 2-thione. The chemical shift of the thiocarbonyl carbon (δ 206.6) in the $^{13}$C NMR was comparable to that of 2-indolinethione (31) (δ 203.9). Compounds with a 2-oxo-indolyl unit such as spirobrassinin (71) and methyl 2-oxotryptamine dithiocarbamate (116) usually give intense fragment ions containing oxygen. On the basis of the spectroscopic data, structure (142) (Figure 2.1) is proposed for the cyclobassinin homologue metabolite.

2.3.4 Antifungal Activity

In bioassays with P. lingam virulent and avirulent isolates cyclobassinin homologue was found to have similar antifungal activity as cyclobassinin. Methyl tryptamine carbamate (4) and N$_b$-carbomethoxyindole-3-methanamine (5) were much less active than methyl tryptamine dithiocarbamate (3). In general the oxygen analogues were much less antifungal compared to their sulfur analogues. The results of bioassays of methyl tryptamine carbamate, N$_b$-carbomethoxyindole-3-methanamine and cyclobassinin homologue against P. lingam isolates are presented in Tables 2.11 and 2.12. At concentrations of 1 x 10$^{-4}$ M and 5 x 10$^{-4}$ M there was no apparent inhibition of mycelial growth in liquid fungal cultures incubated with the carbamates. The mycelial growth was similar to that of the control cultures.

2.3.5 Effect on Phytotoxin Production

Cyclobassinin homologue (6), methyl tryptamine carbamate (4) and N$_b$-carbomethoxyindole-3-methanamine (5) had no effect on the production of nonselective phytotoxins by virulent (BJ-125) isolates of P. lingam. The effect on the host selective phytotoxin phomalide (7) was not observed as the compounds were added to 48-hour-old cultures when the fungus was already producing the toxin.
**Table 2.11** Bioassay of methyl tryptamine carbamate (4) $N_b$-carbomethoxyindole-3-methanamine (5) and cyclobrassinin homologue (6) against virulent *P. lingam* isolate BJ-125 after 72 hours incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>% Inhibition $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl tryptamine carbamate (4)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>6</td>
</tr>
<tr>
<td>$N_b$-carbomethoxyindole-3-methanamine (5)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>7</td>
</tr>
<tr>
<td>Cyclobrassinin homologue (6)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>21</td>
</tr>
</tbody>
</table>

$^a$ % inhibition = 100 - [(growth in treated / growth in control) × 100]; results are the mean of at least three separate experiments, standard deviation ± 0.5

**Table 2.12** Bioassay of methyl tryptamine carbamate (4) $N_b$-carbomethoxyindole-3-methanamine (5) and cyclobrassinin homologue (6) against avirulent *P. lingam* isolate Unity after 72 hours incubation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>% Inhibition $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl tryptamine carbamate (4)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>1</td>
</tr>
<tr>
<td>$N_b$-carbomethoxyindole-3-methanamine (5)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>4</td>
</tr>
<tr>
<td>Cyclobrassinin homologue (6)</td>
<td>$1 \times 10^{-4}$ M</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^{-4}$ M</td>
<td>6</td>
</tr>
</tbody>
</table>

$^a$ % inhibition = 100 - [(growth in treated / growth in control) × 100]; results are the mean of at least three separate experiments, standard deviation ± 0.5
CHAPTER 3: CONCLUSIONS

*Phoma lingam* virulent isolates BJ-125 and ENG-53 biotransformed methyl tryptamine dithiocarbamate (3), a homologue of the phytoalexin brassinin (1) to yield eight metabolites identified as tryptamine (112), N₆-acetyltryptamine (113), methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (114), methyl tryptamine dithiocarbamate-S-oxide (115), methyl 2-oxotryptamine dithiocarbamate (116), indole-3-acetic acid (117), methyl indole-3-acetate (118), and oxindole-3-acetic acid (119). Further transformation of methyl 2-oxotryptamine dithiocarbamate (116) yielded metabolite 120. Compounds 114, 115, 116 and 120 do not appear to have been described before. As part of characterization and proof of structural assignments the synthesis of compounds 114-116 was also accomplished. Particularly noteworthy is the unprecedented rearrangement of methyl tryptamine dithiocarbamate-S-oxide (115) under acid catalysis to yield methyl 2-oxotryptamine dithiocarbamate (116).

*Phoma lingam* avirulent isolate Unity biotransformed methyl tryptamine dithiocarbamate (3) to yield five metabolites which were identified as tryptamine (112), N₆-acetyltryptamine (113), indole-3-acetic acid (117), tryptophol (121) and indole-3-carboxylic acid (104).

The bioassays of methyl tryptamine dithiocarbamate (3) and metabolites against *P. lingam* virulent and avirulent isolates indicated methyl tryptamine dithiocarbamate to be much more toxic than any of its metabolites. This leads to the conclusion that the biotransformations are detoxification processes. Comparison with the phytoalexin brassinin (1) revealed methyl tryptamine dithiocarbamate (3) to be a stronger antifungal agent.
Virulent and avirulent isolates of *P. lingam* showed significant differences in their metabolism and detoxification of methyl tryptamine dithiocarbamate (3). The virulent isolates biotransformed methyl tryptamine dithiocarbamate (3) via three distinct pathways. The avirulent isolate on the other hand biotransformed methyl tryptamine dithiocarbamate (3) through one major pathway. The biotransformation was faster with avirulent isolate Unity (3-5 days) than with virulent isolates BJ-125 and ENG-53 (10-20 days). These differences in the metabolism of methyl tryptamine dithiocarbamate also suggest that the virulent and avirulent isolates of *P. lingam* belong to different species. Generally the biotransformations were faster in the dark than in the light.

The fungal pathogen *A. brassicae* biotransformed methyl tryptamine dithiocarbamate (3) to give two metabolites identified as tryptamine (112) and N₆-acetyltryptamine (113). In contrast to the biotransformations with *P. lingam* isolates this biotransformation did not proceed further to yield the acids.

*Phoma lingam* virulent isolate BJ-125 biotransformed the cruciferous phytoalexin cyclobrassinin (2) to yield one metabolite identified as dioxibrassinin (36). *P. lingam* avirulent isolate Unity on the other hand biotransformed cyclobrassinin (2) to yield two metabolites identified as brassilexin (29) and 3-methylenaminoindole-2-thione (34).

Bioassays of cyclobrassinin and its metabolite against *P. lingam* virulent isolate BJ-125 revealed that cyclobrassinin was more active than the metabolite, dioxibrassinin. However, bioassays of cyclobrassinin against avirulent *P. lingam* isolate Unity showed the metabolites brassilexin and 3-methylenaminoindole-2-thione to be more active than cyclobrassinin. Brassilexin and 3-methylenaminoindole-2-thione were further transformed to undetermined polar and less toxic products. Overall, the biotransformations of cyclobrassinin by *P. lingam* isolates are detoxification processes.

The root rot fungus, *R. solani* biotransformed cyclobrassinin (2) to yield three metabolites identified as 2-mercaptoindole-3-carboxaldehyde (32), the cruciferous phytoalexin brassicanal A (52) and 5-hydroxybrassicanal A (129). In bioassays
cyclobrassicin was found to be more antifungal than any of its metabolites. This indicated that the biotransformation is also a detoxification process.

In the course of characterizing and proving structural assignments, the syntheses of 2-mercaptoindole-3-carboxaldehyde (32) and 3-methylenaminoindole-2-thione (34) were accomplished. These two compounds proved to be key intermediates in the synthesis of the cruciferous phytoalexins brassicanal A (52) and brassilexin (29), respectively.

The fungal pathogens *R. solani* and *P. lingam* metabolized and detoxified the phytoalexin cyclobrassicin (2) via the phytoalexins dioxibrassicin (36), brassicanal A (52) and brassilexin (29), depending on the particular isolate. Since phytoalexins are produced *de novo* by plants upon exposure to microorganisms or other forms of stress, the plants must have a mechanism of getting rid of them when they are no longer required. The results of biotransformations suggest that the fungal pathogens *P. lingam* and *R. solani* can metabolize the phytoalexin cyclobrassicin by utilizing pathways that may operate in the plant. Considering that fungal pathogens have been coevolving with plants for multiple generations, the detoxification of phytoalexins by mimicry appears quite plausible (Pedras and Okanga, 1998c).

Methyl tryptamine carbamate (4) underwent little (< 10%) biotransformation on incubation with virulent *P. lingam* isolate BJ-125 to yield indole-3-acetic acid (117). On incubation with avirulent *P. lingam* isolate Unity, methyl tryptamine carbamate (4) was biotransformed to yield two metabolites identified as tryptophol (121) and indole-3-carboxylic acid (104).

Cyclobrassicin homologue (6) was biotransformed by both virulent and avirulent *P. lingam* isolates to yield mainly one metabolite to which structure 142 was assigned.

Although *N*-carbomethoxyindole-3-methanamine (5) was biotransformed by *P. lingam* isolate BJ-125, no metabolites were detected or isolated. *P. lingam* avirulent isolate Unity on the other hand did not metabolize this compound.
With the exception of indole-3-acetic acid (117), metabolites of methyl tryptamine carbamate (4) and cyclobrassinin homologue (6) were less active against *P. lingam* isolates compared to the parent compounds.

The results of biotransformations of cyclobrassinin (2), methyl tryptamine dithiocarbamate (3), methyl tryptamine carbamate (4), \( N_b \)-carbomethoxyindole-3-methanamine (5) and cyclobrassinin homologue (6) suggest that both the indole and the dithiocarbamate structural units are important for high antifungal activity. Compounds possessing the dithiocarbamate structural unit but lacking the indole unit such as methyl 2-oxotryptamine dithiocarbamate (116), methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (114) were found to be much less active than methyl tryptamine dithiocarbamate. Similarly, compounds possessing the indole unit but lacking the dithiocarbamate unit such as methyl tryptamine dithiocarbamate-S-oxide (115) and indole-3-acetic acid (117) were less active. Cyclobrassinin (2) and cyclobrassinin homologue (6) were less antifungal than their corresponding open-chain analogues, brassinin (1) and methyl tryptamine dithiocarbamate (3). The oxygen-containing analogues methyl tryptamine carbamate (4) and \( N_b \)-carbomethoxyindole-3-methanamine (5) were much less fungitoxic compared to brassinin and methyl tryptamine dithiocarbamate.

The *in vitro* biotransformations have demonstrated that fungal pathogens are able to metabolize and detoxify phytoalexins and analogues. These could be used as models toward understanding the role and fate of the phytoalexins in the *in vivo* plant-pathogen interactions.
CHAPTER 4 : EXPERIMENTAL

4.1 General methods

All chemicals were purchased from Aldrich Chemical Co., Inc., Madison, WI, or Sigma Chemical Co., St. Louis, MO. All solvents were used as such except chloroform and dichloromethane which were redistilled. Analytical thin layer chromatography (TLC) was carried out on precoated silica gel TLC plates (Merck, 60 F$_{254}$, 2.5 × 5.0 cm, 0.25 mm layer thickness) and after elution with a suitable solvent system, examined under UV light (254 / 366 nm). The plates were dipped in a 5% (w / v) aqueous phosphomolybdic acid solution containing 1% (w / v) ceric sulfate and 4% (v / v) H$_2$SO$_4$, followed by heating on a hot plate. Preparative thin layer chromatography (PTLC) was performed on silica gel plates (Merck, 60 F$_{254}$, 20 cm × 20 cm, 0.25 or 0.5 mm thickness). Flash column chromatography (FCC) was performed on silica gel, Merck grade 60, mesh size 230–400, 60 Å. High performance liquid chromatography (HPLC) analysis was carried out with a high performance Hewlett Packard liquid chromatograph equipped with quaternary pump, automatic injector, and diode array detector (wavelength range 190-600 nm), degasser, and hypersil ODS column (5 μM particle size silica, 200 mm × 4.6 mm internal diameter), equipped with an in-line filter. A gradient elution [CH$_3$CN-H$_2$O (25 : 75)-CH$_3$CN (100)], for 35 min., linear gradient, and a flow rate 1.0 mL / min. was used. Infrared spectra were recorded on a Bio-Rad FTS-40 spectrometer. Spectra were measured by the diffuse reflectance method on samples dispersed in KBr. $^1$H NMR spectra were obtained at 300 MHz or 500 MHz with Bruker AMX 300 or AMX 500 spectrometers. Chemical shifts (δ) are reported in parts per million (ppm) relative to TMS. The δ values were referenced to CHCl$_3$ (7.27 ppm), CHDCl$_2$ (5.32 ppm), CD$_2$HCN
(1.94 ppm), CHD$_2$SOCD$_3$ (2.50 ppm), or CHD$_2$OD (3.31 ppm). First-order behaviour was assumed in analysis of $^1$H NMR spectra and multiplicities are indicated by one or more of the following s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. Spin coupling constants (J values) are reported to the nearest 0.5 Hz. $^{13}$C NMR data were collected on a Bruker AMX 300 spectrometer at 75.5 MHz or a Bruker AMX 500 spectrometer at 125.8 MHz. The $^{13}$C chemical shifts (δ values) were referenced to CDCl$_3$ (77.2 ppm), CD$_2$Cl$_2$ (54.0 ppm), CD$_3$CN (118.7 ppm), (CD$_3$)$_2$SO (39.5 ppm), or CD$_3$OD (49.2 ppm). The multiplicity of $^{13}$C signals refers to the number of attached protons (i.e., s = C, d = CH, t = CH$_2$, q = CH$_3$) and were determined based upon HMQC experiments (compounds 114, 116, 129 and 140), chemical shift and consistency within a series of similar structures, as well as relative intensity of each signal. Mass spectra (MS) [high resolution (HR), electron impact (EI), or chemical ionization (CI) with ammonia as ionizing gas] were obtained on a VG 70 SE mass spectrometer, employing a solids probe. Specific rotations, [α]$_D$ were determined at ambient temperature on a Perkin-Elmer 141 polarimeter using a 1 mL, 10 cm path length cell.

Fungal cultures: fungal isolates were obtained from Agriculture and Agri-Food Canada Research Station, Saskatoon Saskatchewan. The virulent blackleg (P. lingam) isolates BJ-125, ENG-53, avirulent isolate Unity (now considered a different species), R. solani isolate AG2-1 and A. brassicaceae isolates IBJ and AB3 were employed in these studies. The P. lingam and A. brassicaceae fungal isolates were grown on V8 agar [ 20% (v/v) V8 juice, 0.75g / L CaCO$_3$, 100 mg / L streptomycin sulphate, 40 mg / L Rose Bengal, 15 g / L agar ] plates at 25 °C under continuous light for 14-21 days. Spore suspensions of each isolate were prepared by overlaying the V8 agar plates with 10 mL sterile distilled water and the plate surfaces were rubbed with a flamed rod to dislodge the pycnidiospores (herein called spores). The suspension was filtered and transferred to centrifuge tubes and the spores were separated by centrifugation at 3000g for ten minutes. After washing once with sterile distilled water the spores were stored in 100 μL quantities
at -20 °C. Spores were counted using a hemocytometer. Liquid cultures of the fungi were started by inoculating minimal medium (Tinline et al., 1960) or potato dextrose broth (PDB) medium (Atlas and Parks, 1993) with spores. R. solani was grown on potato dextrose agar (PDA). Solid cultures were started by placing a plug of mycelia 5 mm diameter at the center of the plate containing the agar. Liquid cultures were initiated by cutting plugs of mycelia from the edges of 5 or 6-day-old cultures and placing them in potato dextrose broth medium (5 plugs per 100 mL of medium).

Extraction of fungal cultures: each sample was extracted with diethyl ether to give a neutral extract. After the first extraction the aqueous layer was acidified to pH 2 with concentrated HCl and again extracted with ether to yield an acidic extract. Finally the aqueous layer was basified to pH 10 with concentrated ammonia solution (29%) and extracted with CHCl₃ [containing 2% NH₄OH (v/v)] to yield a basic extract. The extracts were dried over Na₂SO₄, evaporated at reduced pressure and analyzed by TLC and HPLC. The samples to be analyzed by HPLC were dissolved in acetonitrile and filtered through a tight cotton plug.

4.2 Methyl tryptamine dithiocarbamate

4.2.1 Time-course experiments

4.2.1.1 Biotransformation of methyl tryptamine dithiocarbamate by Phoma lingam isolates BJ-125 and Unity

**Typical time course procedure:** four 250 mL Erlenmeyer flasks each containing 100 mL of minimal medium were employed. Three of the flasks were each inoculated with spores (2.0 × 10⁹ spores) of P. lingam isolate ENG-53, BJ-125 or Unity. The flasks were incubated at 25 ± 2 °C on a shaker at 150 rpm. After 48 hours solutions of methyl tryptamine dithiocarbamate (3, final concentration 2.0 × 10⁻⁴ M) in DMSO [final concentration 0.5% (v/v)] were added to fungal cultures in two of the flasks and to
uninoculated medium (control) in another flask. To fungal cultures (control) in the third flask 500 μL of DMSO was added. Samples (5 mL each) were taken from the flasks immediately after adding the compounds. Subsequently 10 mL samples were taken after 1, 2, 3, 5, 7, 14 days and final samples after 21 days. The samples were either immediately extracted or frozen for later extraction. The 5 mL and 10 mL samples were extracted with 10 mL and 20 mL of solvent, respectively, as described in section 4.1. After evaporation of the solvent each residue was dissolved in 1 mL of acetonitrile and filtered through a tight cotton plug into a HPLC vial for analysis.

After 21 days fungal cultures were filtered to separate mycelium from the broth. Each broth (35 mL) was extracted with an equivalent amount of solvent to give neutral, acidic and basic extracts as described in section 4.1. The mycelium from each flask was air dried, ground to a powder and extracted with diethyl ether (2 × 20 mL). The contents of the uninoculated flask were similarly extracted to furnish neutral, acidic and basic extracts. Each extract was dried over Na₂SO₄ and the solvent evaporated under reduced pressure. After TLC analyses the residues were each dissolved in 2 mL acetonitrile and filtered through a tight cotton plug into HPLC vial. The sample was diluted further for HPLC analysis by adding 500 μL acetonitrile.

4.2.1.2 Biotransformation of methyl tryptamine dithiocarbamate by Alternaria brassicae isolates AB3 and IBJ

Typical time-course procedure: four 250 mL Erlenmeyer flasks each containing 100 mL of minimal or potato dextrose medium were employed. Three of the flasks were inoculated with spores (2.4 × 10⁵) of A. brassicae isolate AB3 or IBJ. The flasks were incubated at 25 ± 2 °C on a shaker at 150 rpm. After 48 hours solutions of methyl tryptamine dithiocarbamate (3, final concentration 1.0 × 10⁻⁴ M) in DMSO [final concentration 0.5% (v/v)] were added to fungal cultures in two of the flasks and to uninoculated medium (control) in another flask. To fungal cultures (control) in the third
flask 500 µL of DMSO was added. Samples (5 mL each) were taken from the flasks immediately after adding the compounds. Subsequently, 10 mL samples were taken after 1, 2, 3, 5, 7, 14 days and final samples after 21 days. The 5 mL and 10 mL samples were extracted with 10 mL and 20 mL of solvent, respectively, as described in section 4.1. After evaporation of the solvent each residue was analyzed by HPLC as described in section 4.2.1.1.

After 21 days fungal cultures were filtered to separate mycelium from the broth. Each broth (35 mL) was extracted with an equivalent amount of solvent to give neutral, acidic and basic extracts as described in section 4.1. The contents (35 mL) of the uninoculated flask were similarly extracted to furnish neutral, acidic and basic extracts. The air dried and ground mycelia from each flask was extracted as described in section 4.2.1.1. All the extracts were processed and analyzed by HPLC as described in section 4.2.1.1.

4.2.2 Scale up experiments: isolation of metabolites

4.2.2.1 Biotransformation of methyl tryptamine dithiocarbamate by Phoma lingam virulent isolates BJ-125 and ENG-53

**Typical scale up procedure:** twelve 250 mL Erlenmeyer flasks each containing 100 mL of minimal medium were employed. Eleven of the flasks were inoculated with spores of *P. lingam* isolate ENG-53. After 48 hours solutions of methyl tryptamine dithiocarbamate (3, final concentration 2.0 × 10⁻⁴ M) in DMSO [final concentration 0.5% (v/v)] were added to fungal cultures in ten flasks and to uninoculated medium in one flask. To the fungal cultures (control) in the eleventh flask 500 µL of DMSO was added. Two test flasks and the control flask were chosen for sampling. The biotransformation was monitored by taking 5 and 10 mL samples over a period of 14 days. The 5 and 10 mL samples were extracted and analyzed as described in section 4.2.1.1.
After 14 days the cultures were filtered to separate mycelium from the broth. The combined broth (910 mL) from the ten test flasks was divided into two equal portions. Each was then extracted with an equivalent amount of solvent to yield neutral and acidic extracts as described in section 4.1. The broth (55 mL) from the control flask was similarly extracted. The combined mycelia from the test flasks was air dried, ground in a mortar and extracted with ether (2 × 50 mL). The air dried mycelia from the control flask was similarly extracted with ether (2 × 20 mL). The diethyl ether extracts were each dried over Na₂SO₄ and the solvent evaporated under reduced pressure. Each of the residues from both the broth and mycelia extracts was analyzed by TLC and HPLC.

Preparative TLC [CH₂Cl₂-MeOH (97 : 3)] was carried out on the broth neutral extract (58.9 mg). This resulted in the isolation of N₈-acetyltryptamine (113, 4.4 mg), methyl 2-oxotryptamine dithiocarbamate (116, 1.7 mg), methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (114, 9.7 mg), methyl tryptamine dithiocarbamate-S-oxide (115, 1.9 mg) and recovered methyl tryptamine dithiocarbamate (3, 9.3 mg).

Similarly, preparative TLC [CH₂Cl₂-MeOH (95 : 5)] was carried out on the broth acidic extract (75.3 mg). Indole-3-acetic acid (117, 4.8 mg) and methyl indole-3-acetate (118, 1.0 mg) were isolated.

Detection and isolation of tryptamine in the biotransformation of methyl tryptamine dithiocarbamate: four 250 mL Erlenmeyer flasks each containing 100 mL of minimal medium were employed in this study. Three of the flasks were inoculated with spores of P. lingam isolate BJ-125. After 48 hours solutions of methyl tryptamine dithiocarbamate (3, final concentration 1.6 × 10⁻⁴ M) in DMSO [final concentration 0.5 % (v/v)] were added to fungal cultures in three of the flasks. To fungal cultures (control) in the fourth flask 500 µL of DMSO was added. One test flask and the control flask were chosen for sampling. Samples (5 mL each) were taken immediately after adding the compounds. Subsequently, the biotransformation was monitored by
taking 10 mL samples over a period of 5 days. The 5 and 10 mL samples were extracted following a typical time course procedure described in section 4.2.1.1. After evaporation of the solvent each residue was analyzed by TLC [CHCl₃-MeOH-NH₄OH (80: 20: 1)].

After 5 days the contents of the three test flasks were filtered to separate mycelium from the broth. The combined broth (275 mL) was extracted with diethyl ether (275 mL) to give a neutral extract. The broth was then basified to pH 10 with concentrated ammonia solution and extracted with CHCl₃-NH₄OH (98 : 2) (275 mL) to yield a basic extract. Both extracts were dried over Na₂SO₄, the solvent evaporated under reduced pressure and the residues analyzed by TLC [CHCl₃-MeOH-NH₄OH (80 : 20 : 1)]. The contents of the control flask were similarly extracted to yield neutral and basic extracts. These were analyzed by TLC and compared with the test samples.

Preparative TLC [CHCl₃-MeOH-NH₄OH (80 : 20 : 1)] on the basic extract (111.4 mg) yielded 1.5 mg of tryptamine (112).

**Further biotransformation of metabolites:** each of the metabolites tryptamine (113), N₈-acetyltryptamine (113), methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (114), methyl tryptamine dithiocarbamate-S-oxide (115), methyl 2-oxotryptamine dithiocarbamate (116), indole-3-acetic acid (117), methyl indole-3-acetate (118) and oxindole-3-acetic acid (119) was subjected to further biotransformation by virulent isolates of *P. lingam*. For each compound a final solution concentration of 2 × 10⁻⁴ M was generally employed. If further biotransformation led to detection of new compounds, i.e. not detected in the biotransformation of methyl tryptamine dithiocarbamate, scale up experiments were conducted to isolate and characterize them.

**Isolation of the metabolites of methyl 2-oxotryptamine dithiocarbamate:** nineteen 250 mL Erlenmeyer flasks each containing 100 mL of minimal medium were employed in a scaled up procedure. All the flasks were inoculated with spores of *P. lingam* isolate BJ-125. After 48 hours solutions of methyl 2-
oxotryptamine dithiocarbamate (116, final concentration $1.9 \times 10^4$ M) in DMSO [final concentration 0.5 % (v/v)] were administered to fungal cultures in eighteen of the flasks. To fungal cultures in the remaining flask (control) 500 μL of DMSO was added. Samples (10 mL each) were taken from three of the test flasks and the control after 2, 8, 13 days, and final samples after 14 days.

After 14 days the contents of the test flasks were filtered to separate mycelium from the broth. The combined broth (1720 mL) was divided into three equal portions. Each was extracted with diethyl ether (600 mL) to give three neutral and three acidic extracts as described in section 4.1. The three neutral extracts were combined, dried over Na₂SO₄ and the solvent evaporated under reduced pressure. The three acidic extracts were combined and treated similarly. The residues were analyzed by TLC and HPLC. The contents of the control flask were similarly filtered after 14 days to separate mycelium from the broth. The broth (70 mL) was extracted with ether (70 mL each) to furnish neutral and acidic extracts. These were analyzed by TLC, HPLC and compared with the test samples.

Flash column chromatography [silica gel ca. 40 mL, CH₂Cl₂-MeOH (95 : 5)] on the neutral extract (314 mg) afforded six fractions (each ca. 25 mL); the sixth fraction (39 mg, most polar) was further fractionated by preparative TLC [ethyl acetate-hexane (80 : 20)]. This resulted in the isolation of methyl 3-hydroxy-2-oxotryptamine dithiocarbamate (120, 9.2 mg).

4.2.2.2 Biotransformation of methyl tryptamine dithiocarbamate by Phoma lingam avirulent isolate Unity

Typical scale up procedure: seven 250 mL Erlenmeyer flasks each containing 100 mL of minimal medium were used. Six flasks were inoculated with spores of P. lingam isolate Unity. After 48 hours solutions of methyl tryptamine dithiocarbamate (3, final concentration $1.6 \times 10^4$ M) in DMSO [final concentration 0.5% (v/v)] were administered to fungal cultures in five flasks and to uninoculated medium (control) in
another flask. To the fungal cultures in the sixth flask (control) 500 µL of DMSO was added. The biotransformation was monitored by taking 10 mL samples over a period of 4 days.

After 4 days the contents of the five test flasks were filtered to separate mycelium from the broth. The combined broth (490 mL) was extracted with diethyl ether (500 mL) to give neutral, and acidic extracts as described in section 4.1. Both extracts were dried over Na₂SO₄ and the solvent evaporated under reduced pressure. The residues were analyzed by TLC and HPLC. The contents of the two control flasks were similarly extracted after 4 days to yield neutral and acidic extracts. These were analyzed by TLC, HPLC, and compared with the test samples.

Preparative TLC [CH₂Cl₂-MeOH (96 : 4)] on the neutral extract (21 mg) led to the isolation of N₆-acetyltryptamine (113, 1.8 mg), indole-3-acetic acid (117, 2.2 mg), and tryptophol (121, 2.9 mg). Tryptamine (112) was detected and isolated following a similar procedure as for the virulent isolates of *P. lingam* (section 4.2.2.1).

**Further biotransformation of metabolites:** each of the metabolites tryptamine (112), N₆-acetyltryptamine (113), indole-3-acetic acid (117), tryptophol (121) and indole-3-carboxylic acid (104) was subjected to further biotransformation by avirulent isolates of *P. lingam*. In each case a typical time-course procedure was followed. For each compound final solution concentrations of $2 \times 10^{-4}$ M were generally employed.

### 4.2.2.3 Biotransformation of methyl tryptamine dithiocarbamate by *Alternaria brassicae*

Four 250 mL Erlenmeyer flasks each containing 100 mL of PDB medium were employed. Two of the flasks were inoculated with spores of *A. brassicae* isolate AB3 while the other two were inoculated with *A. brassicae* isolate IBJ. After 48 hours solutions of methyl tryptamine dithiocarbamate (3, final concentration $1.0 \times 10^{-4}$ M) in DMSO [final concentration 0.5 % (v / v)] were added to fungal cultures in two of the
flasks (one each of AB3 and IBJ). To the fungal cultures (controls) in the other two flasks (one each of AB3 and IBJ) 500 µL of DMSO was added. Samples (10 mL) of each were taken immediately after adding the compounds. Subsequently, 10 mL samples were taken after 1, 2, 5 days and final samples after 5 days.

After 5 days fungal cultures were filtered to separate mycelium from the broth. Each broth (60 mL) was extracted with diethyl ether (60 mL), the extract dried over Na₂SO₄ and the solvent evaporated under reduced pressure. The residue was then analyzed by TLC and HPLC. For each of the isolates AB3 and IBJ preparative TLC [CH₂Cl₂-MeOH (95 : 5)] on the residue resulted in the isolation of one metabolite which was identified as N₆-acetyltryptamine (113, 1.2 mg each). Tryptamine was detected and isolated following a similar procedure as for the virulent P. lingam biotransformation (section 4.2.2.1).

Biotransformations in minimal medium yielded identical metabolites, but the fungus growth was slower.

4.2.3 Bioassays

Spore germination and radial growth of mycelia: six- and twelve-well tissue culture plates were employed for the bioassay studies. Solutions of the test compound (final concentration 5 × 10⁻⁴ M and 1 × 10⁻⁴ M) in DMSO (final concentration 2%) were prepared and added to V8 juice agar medium (10 mL) at ca. 50 °C in a Falcon tube. The agar medium and the compound were quickly mixed (vortex mixer) and poured into three wells on a six-well tissue culture plate. A control medium containing only DMSO and the V8 agar was poured into the remaining three wells on the plate. After preparation the plates were allowed to set for at least one hour before inoculation with fungal spores. A suspension of spores (2 × 10⁸ / mL) in water was prepared for each fungal isolate to be assayed. Inoculation was done by adding a 5 µL drop of fungal spores to the center of each well on the culture plate. The plates were then incubated under constant light at 25 ± 2 °C for seven days. After spore germination the diameter of the
mycelial covered area was measured daily over a period of one week. Each bioassay was repeated at least three times. The twelve-well plates were used while assaying several compounds at the same time.

4.2.4 Synthesis of compounds

**Methyl tryptamine dithiocarbamate (3)**

![Chemical structure](image)

Carbon disulfide (160 μL, 2.8 mmol) was added to a mixture of tryptamine (112, 320 mg, 2 mmol), triethylamine (360 μL), and pyridine (2 mL) at 0 °C and the mixture stirred at 0 °C for 1 hour. Methyl iodide (160 μL, 2.6 mmol) was added and the reaction mixture kept at 3 °C in the refrigerator for 15 hours. The reaction mixture was then poured into 1.5 M H₂SO₄ (40 mL) and the crude product extracted with diethyl ether (3 × 50 mL). The combined extracts were successively washed with water (150 mL), saturated NaHCO₃ (150 mL), water (150 mL) and the ethereal solution dried over Na₂SO₄. Evaporation of the solvent under reduced pressure yielded 511 mg of crude product. Fractionation by flash column chromatography (CH₂Cl₂) yielded pure methyl tryptamine dithiocarbamate (3, 454 mg, 91%).
$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.07 (br s, 1H, D$_2$O exchangeable), 7.62 (d, $J = 8$ Hz, 1H), 7.37 (d, $J = 8$ Hz, 1H), 7.22 (ddd, $J = 8, 8, 1$ Hz, 1H), 7.14 (ddd, $J = 8, 8, 1$ Hz, 1H), 7.02 (s, 1H), 7.00 (br s, 1H, D$_2$O exchangeable), 4.04-4.07 (m, 2H), 3.08-3.12 (m, 2H), 2.56 (s, 3H), and minor signals (ca. 1/4 intensity of the major ones) due to a rotamer at $\delta$ 7.57 (d, $J = 8$ Hz), 3.74 (m), 2.68 (s).

$^{13}$C NMR (125.8 MHz, CDCl$_3$) $\delta$ 198.8 (s), 136.4 (s), 127.2 (s), 122.5 (d), 122.4 (d), 119.7 (d), 118.7 (d), 112.3 (s), 111.4 (d), 47.2 (t), 24.0 (t), 18.1 (q) and minor signals (ca. 1/4 intensity of the major ones) due to a rotamer at $\delta$ 201.7 (s), 126.9 (s), 118.5 (d), 46.1 (t), 24.6 (t), 19.0 (q).

FTIR (cm$^{-1}$) 3408, 3324, 2915, 1501, 1455, 1338, 1090, 934, 744.

EIMS $m/z$ (%) 250 (M$^+$, 6), 202 (68), 143 (63), 130 (100).

FABMS $m/z$ (%) 251 (M$^+$ + 1, 47), 144 (100).

HREIMS $m/z$ calc. for C$_{12}$H$_{14}$N$_2$S$_2$ (250.0598), found 250.0600.
N\textsubscript{6}-acetyltryptamine (113)

\[
\begin{align*}
\text{112} & \rightarrow \text{113}
\end{align*}
\]

Acetic anhydride (200 \textmu L, 2 mmol) and Et\textsubscript{3}N (100 \textmu L) were added to a solution of tryptamine (112, 320 mg, 2 mmol) in THF (4 mL) and the mixture stirred at room temperature for 1 hour. The solvent was evaporated under reduced pressure and the residue dissolved in 10 mL CH\textsubscript{2}Cl\textsubscript{2}. This solution was washed with saturated NaHCO\textsubscript{3} (10 mL) and water (10 mL), respectively. The organic layer was then dried over Na\textsubscript{2}SO\textsubscript{4} and the solvent removed under reduced pressure to give 490 mg of crude product. Preparative TLC on a portion (180 mg) of the crude product yielded N\textsubscript{6}-acetyltryptamine (113, 118 mg), 80 \% yield from tryptamine.

\textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) \textgreek{d} 8.44 (br s, 1H, D\textsubscript{2}O exchangeable), 7.57 (d, J = 8 Hz, 1H), 7.35 (d, J = 8 Hz, 1H), 7.18 (ddd, J = 8, 8, and 1 Hz, 1H), 7.10 (ddd, J = 8, 8, and 1 Hz, 1H), 6.99 (s, 1H), 5.78 (br s, 1H, D\textsubscript{2}O exchangeable), 3.57 (m, 2H), 2.95 (t, J = 7 Hz, 2H), 1.90 (s, 3H).

\textsuperscript{13}C NMR (75.5 MHz, CDCl\textsubscript{3}) \textgreek{d} 170.4 (s), 136.5 (s), 127.4 (s), 122.1 (d, 2 carbons), 119.4 (d), 118.6 (d), 112.8 (s), 111.4 (d), 40.0 (t), 25.2 (t), 23.2 (q).
FTIR (cm⁻¹) 3398, 3292, 2923, 2851, 1653, 1636, 1558, 1540, 1456, 743.

EIMS m/z (%) 202 (M⁺, 25), 143 (100), 130 (96).

CIMS m/z (%) 203 (M⁺ + 1, 100), 143 (42), 130 (26).

HREIMS m/z calcd. for C₁₂H₁₄N₂O (202.1106), found 202.1105.

**Methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (114)**

![Chemical Structure](image)

Methyl tryptamine dithiocarbamate (3, 20 mg, 0.08 mmol) and Rose Bengal (8 mg, 0.008 mmol) were dissolved in 5 mL of 5% pyridine-methanol. The solution was transferred to a Pyrex test tube and irradiated with light from a 100 W halogen bulb. Air was continuously bubbled through the solution while cooling in ice-water. The irradiation was done for 2 hours while monitoring the reaction by TLC. After 2 hours the reaction mixture was transferred to a round bottom flask, dimethyl sulfide (200 μL) added and the solution stirred at room temperature for another two hours. An equivalent amount (5 mL) of toluene was added and the solvent evaporated under reduced pressure. The residue was
further dissolved in 10 mL of toluene and the solvent again evaporated under reduced pressure. Preparative TLC [CH$_2$Cl$_2$-MeOH (98 : 2)] on the crude product yielded methyl 3a-hydroxy-3,3a,8,8a-tetrahydropyrrolo[2,3-b]indol-1(2H)-yl carbodithioate (114, 12.0 mg, 65%) and starting material (2.8 mg). The % yield was calculated after subtraction of recovered starting material.

$[\alpha]_D = +473$ (c 0.0014, CH$_2$Cl$_2$), for the compound isolated from fungal metabolism of 3.

$^1$H NMR (300 MHz, CD$_2$Cl$_2$) $\delta$ 7.33 (d, $J = 8$ Hz, 1H), 7.18 (ddd, $J = 8$, 8, and 1 Hz, 1H), 6.81 (ddd, $J = 8$, 8, and 1 Hz, 1H), 6.64 (d, $J = 8$ Hz, 1H), 5.71 (s, 1H), 4.06-4.14 (m, 1H), 3.28-3.37 (m, 1H), 2.62 (s, 3H), 2.52-2.59 (m, 2H).

$^{13}$C NMR (75.5 MHz, CDCl$_3$) $\delta$ 197.4 (s), 149.0 (s), 130.9 (d), 128.7 (s), 123.5 (d), 119.5 (d), 110.2 (d), 87.2 (d), 86.2 (s), 50.1 (t), 35.9 (t), 19.3 (q).

FTIR (cm$^{-1}$) 3368, 3361, 2919, 2852, 1610, 1415, 1312, 1183, 1056, 949, 747.

EIMS $m/z$ (%) 266 (M$^+$, 100), 219 (21), 186 (45), 160 (41), 133 (61).

CIMS $m/z$ (%) 267 (M$^+$ + 1, 100), 219 (64), 187 (71), 160 (49), 144 (54), 133 (23).

HREIMS $m/z$ calcd. for C$_{12}$H$_{14}$N$_2$OS$_2$ (266.0548), found 266.0547.
Methyl tryptamine dithiocarbamate-S-oxide (115)

Methyl tryptamine dithiocarbamate (3, 20 mg, 0.08 mmol) was dissolved in 2 mL CH₂Cl₂ and the solution cooled to 0 °C in ice-water. m-CPBA (18 mg, 0.08 mmol) was added, the mixture stirred at 0 °C for 30 minutes followed by addition of dimethyl sulfide (500 μL) and successive washing with saturated NaHCO₃ (2 mL) and H₂O (2 mL). The solvent was evaporated under reduced pressure and the residue separated on TLC [CH₂Cl₂-MeOH (95 : 5)] to yield methyl tryptamine dithiocarbamate-S-oxide (115, 9 mg), 44% yield. The other products identified were methyl 2-oxotryptamine dithiocarbamate (116, 0.2 mg) and recovered starting material (3, 0.6 mg). The % yield was calculated after subtraction of recovered starting material.

¹H NMR (300 MHz, CD₂Cl₂) δ 9.02 (br s, 1H, D₂O exchangeable), 7.57 (d, J = 8 Hz, 1H), 7.36 (d, J = 8 Hz, 1H), 7.16 (ddd, J = 8, 8, 1 Hz, 1H), 7.09 (ddd, J = 8, 8, 1 Hz, 1H), 7.02 (s, 1H), 3.80 (t, J = 7 Hz, 1H), 3.13 (t, J = 7 Hz, 1H), 2.35 (s, 3H).

¹³C NMR (75.5 MHz, CD₂Cl₂) δ 194.5 (s), 137.0 (s), 127.4 (s), 123.4 (d), 122.4 (d), 119.7 (d), 118.6 (d), 111.9 (d), 111.3 (s), 45.8 (t), 25.6 (t), 13.3 (q).
FTIR (cm⁻¹) 3408, 3215, 2920, 2872, 1527, 1514, 1456, 1337, 905, 744.

EIMS m/z (%) 266 (M⁺, 2), 248 (7), 218 (5), 202 (8), 143 (59), 130 (100).

HREIMS m / z calcd. for C₁₂H₁₄N₂S₂O (266.0548), found 266.0559.

**Methyl 2-oxotryptamine dithiocarbamate (116)**

Methyl tryptamine dithiocarbamate (3, 50 mg, 0.2 mmol) was dissolved in 4 mL of CH₂Cl₂ and the solution cooled to 0 °C in ice-water. m-CPBA (52 mg, 0.24 mmol) was added and the mixture stirred at 0 °C for 30 minutes. A solution of 35 mg (0.2 mmol) p-toluene sulfonic acid (p-TsOH) in methanol (0.5 mL) was added, the reaction mixture allowed to warm to room temperature and kept stirring for 3 hours. Dimethyl sulfide (500 μL) was then added and stirring continued for another 15 minutes. The reaction mixture was successively washed with saturated NaHCO₃ (5 mL), water (5 mL) and the solvent evaporated under reduced pressure. The residue was subjected to preparative TLC [CH₂Cl₂-MeOH (97 : 3)] to yield methyl 2-oxotryptamine dithiocarbamate (116, 13 mg, 33% yield) and starting material (3, 13 mg). The % yield was calculated after subtraction of recovered starting material.
$[\alpha]_D = -50$ (c 0.0008, CH$_2$Cl$_2$), for the compound isolated from fungal metabolism of 3.

$^1$H NMR (300 MHz, CD$_2$Cl$_2$) $\delta$ 8.57 (br s, 1H, D$_2$O exchangeable), 8.02 (br s, 1H, D$_2$O exchangeable), 7.22-7.29 (m, 2H), 7.07 (dd, J = 8, 8 Hz, 1H), 6.92 (d, J = 8 Hz, 1H), 4.04-4.10 (m, 1H), 3.84-3.88 (m, 1H), 3.55-3.59 (m, 1H), 2.59 (s, 3H), 2.38-2.45 (m, 1H), 2.09-2.18 (m, 1H).

$^{13}$C NMR (75.5 MHz, CDCl$_3$) $\delta$ 199.0 (s), 180.7 (s), 140.9 (s), 128.9 (s), 128.4 (d), 124.1 (d), 123.0 (d), 110.1 (d), 45.4 (t), 45.0 (d), 28.7 (t), 18.0 (q).

FTIR (cm$^{-1}$) 3234, 3204, 1700, 1619, 1519, 1470, 1335, 943, 751.

EIMS $m/z$ (%) 266 (M$^+$, 3), 218 (67), 159 (10), 146 (100), 133 (40).

CIMS $m/z$ (%) 267 (M$^+$ + 1, 4), 219 (100), 146 (35), 130 (8).

HREIMS $m/z$ calcd. for C$_{12}$H$_{14}$N$_2$OS$_2$ (266.0548), found 266.0550.
Indole-3-acetic acid (117, 0.50 g, 0.0029 mol) was dissolved in dimethyl sulfoxide (1 mL, 0.014 mol) and concentrated HCl (1 mL, 0.0125 mol) added dropwise with stirring. The reaction mixture was stirred at room temperature for 4 hours, diluted with water (4 mL), neutralized with concentrated ammonia (29%) solution and extracted with ethyl acetate (4 x 25 mL). The combined ethyl acetate (100 mL) extracts were washed with water (100 mL), dried over Na₂SO₄ and the solvent evaporated under reduced pressure. Flash column chromatography [CH₂Cl₂-MeOH (95 : 5)] on the residue followed by crystallization from benzene-acetone afforded 290 mg (53% yield) of oxindole-3-acetic acid (119).

¹H NMR (300 MHz, DMSO-d₆) δ 12.34 (br s, 1H, D₂O exchangeable), 10.39 (s, 1H, D₂O exchangeable), 7.22 (d, J = 8 Hz, 1H), 7.17 (dd, J = 8, 8 Hz, 1H), 6.92 (dd, J = 8, 8 Hz, 1H), 6.81 (d, J = 8 Hz, 1H), 3.62 (dd, J = 6, 6 Hz, 1H), 2.90 (dd, J = 6, 17 Hz, 1H), 2.70 (dd, J = 6, 17 Hz, 1H).
$^{13}$C NMR (125.8 MHz, DMSO-d$_6$) $\delta$ 178.1 (s), 172.1 (s), 142.8 (s), 129.3 (s), 127.6 (d), 123.6 (d), 121.1 (d), 109.1 (d), 41.8 (d), 34.0 (t).

FTIR (cm$^{-1}$): 3263, 2923, 2853, 1711, 1621, 1485, 1470, 1340, 1218, 751.

EIMS $m/z$ (%) 191 $M^+$, 21), 173 (10), 145 (66), 117 (28). CIMS $m/z$ (%) 192 (M$^+$ + 1, 100), 145 (19).

HREIMS $m/z$ calcd for C$_{10}$H$_9$NO$_3$ (191.0582), found 191.0577.
4.2.5 Spectroscopic Data

Tryptamine (112)

\[
\begin{align*}
\text{1H NMR (300 MHz, DMSO-}d_6\text{)} & \delta 10.85 \text{ (br s, 1H, D}_2\text{O exchangeable), 7.48 (d, J = 8} \\
& \text{Hz, 1H), 7.31 (d, J = 8 Hz, 1H), 7.09 (s, 1H), 7.03 (dd, J = 8, 7 Hz, 1H), 6.93 (dd, J} \\
& = 8, 7 \text{ Hz, 1H), 2.79 (m, 4H), 1.60 (br s, 2H, D}_2\text{O exchangeable).}
\end{align*}
\]

\[
\begin{align*}
\text{13C NMR (75.5 MHz, DMSO-}d_6\text{)} & \delta 136.3 \text{ (d), 127.4 (s), 122.6 (d), 120.8 (d), 118.4 (s),} \\
& 118.1 \text{ (d), 112.6 (s), 111.3 (d), 42.8 (t), 29.6 (t).}
\end{align*}
\]

FTIR (cm\(^{-1}\)) 3404, 3345, 2920, 2869, 1590, 1455, 741.

EIMS \text{m/z (%)} 160 (M\(^+\), 17), 130 (100).

HREIMS \text{m/z calcd. for C}_{16}\text{H}_{12}\text{N}_2 \text{ (160.1000), found 160.1004.}
Indole-3-acetic acid (117)

\[
\begin{align*}
\text{OH} \\
\text{N}
\end{align*}
\]

\(117\)

\(^1\text{H}\) NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 12.18 (br s, 1H, D\(_2\)O exchangeable), 10.92 (s, 1H, D\(_2\)O exchangeable), 7.51 (d, \(J = 8\) Hz, 1H), 7.37(d, \(J = 8\) Hz, 1H), 7.24 (d, \(J = 8\) Hz, 1H), 7.09 (m, 1H), 6.97-7.02 (m, 1H), 3.62 (s, 2H).

\(^{13}\text{C}\) NMR (75.5 MHz, DMSO-\(d_6\)) \(\delta\) 173.2 (s), 136.1 (s), 127.2 (s), 123.9 (d), 121.0 (d), 118.6 (d), 118.4 (d), 111.4 (d), 107.6 (s), 31.0 (t).

FTIR (cm\(^{-1}\)) 3391, 1700, 1456, 1406, 1208, 1100, 931, 752.

EIMS \(m/z\) (%) 175 (M\(^+\), 39), 130 (100).

CIMS \(m/z\) (%) 176 (M\(^+ + 1\), 100), 130 (98).

HREIMS \(m/z\) calcd. for C\(_{16}\)H\(_9\)NO\(_2\) (175.0633), found 175.0631.
Methyl indole-3-acetate (118)

\[
\begin{array}{c}
\text{\textbf{118}} \\
\text{\textbf{OCH}_3}
\end{array}
\]

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 8.11 (s, 1H, D$_2$O exchangeable), 7.61 (d, $J = 8$ Hz, 1H), 7.33 (d, $J = 8$ Hz, 1H), 7.11 - 7.24 (m, 3H), 3.78 (s, 2H), 3.70 (s, 3H).

$^{13}$C NMR (75.5 MHz, CDCl$_3$) $\delta$ 172.6 (s), 136.1 (s), 127.2 (s), 123.1 (d), 122.2 (d), 119.7 (d), 118.8 (d), 111.2 (d), 108.4 (s), 52.0 (q), 31.2 (t).

FTIR (cm$^{-1}$) 3408, 2950, 2926, 2843, 1733, 1455, 1435, 1332, 1010, 743.

EIMS $m/z$ (%) 189 (M$^+$, 37), 130 (100). HREIMS $m/z$ calcd. for C$_{11}$H$_{11}$NO$_2$ (189.0790), found 189.0788.
Methyl 3-hydroxy-2-oxotryptamine dithiocarbamate (120)

\[
\text{HO} \quad \text{NH} \quad \text{SCH}_3 \\
\text{NH} \quad \text{O} \\
\text{N}
\]

1H NMR (300 MHz, CD$_3$OD) δ 7.36 (d, J = 8 Hz, 1H), 7.27 (dd, J = 8, 8 Hz, 1H), 7.07 (dd, J = 8, 8 Hz, 1H), 6.89 (d, J = 8 Hz, 1H), 3.60-3.73 (m, 2H), 2.50 (s, 3H), 2.16-2.29 (m, 2H).

13C NMR (125.8 MHz, CD$_3$OD) δ 199.8 (s), 181.6 (s), 142.5 (s), 132.5 (s), 130.7 (d), 125.2 (d), 123.8 (d), 111.4 (d), 76.5 (s), 43.0 (t), 36.6 (t), 17.8 (q).

FTIR (cm$^{-1}$) 3305, 3271, 1715, 1622, 1471, 1188, 754.

EIMS m/z (%) 282 (M$^+$, 5), 264 (7), 234 (15), 202 (8), 165 (15), 149 (100), 146 (25).

FABMS m/z (%) 283 (M$^+$ + 1, 100), 269 (25), 217 (27), 158 (27).

HREIMS m/z calcd. for 282.0497 (C$_{12}$H$_{14}$N$_2$O$_2$S$_2$), found 282.0497.
Tryptophol (121)

\[
\text{1H NMR (300 MHz, CDCl}_3\text{) } \delta 8.04 \text{ (br s, 1H, D}_2\text{O exchangeable), 7.61 (d, J = 7 Hz, 1H), 7.37 (d, J = 7 Hz, 1H), 7.20 (dd, J = 7, 7 Hz, 1H), 7.12 (dd, J = 7, 7 Hz, 1H), 7.08 (s, 1H), 3.90 (t, J = 6 Hz, 2H), 3.03 (t, J = 6 Hz, 2H), 1.85 (br s, 1H, D}_2\text{O exchangeable).}
\]

\[
\text{13C NMR (75.5 MHz, CDCl}_3\text{) } \delta 136.5 \text{ (s), 127.5 (s), 122.4 (d), 122.2 (d), 119.5 (d), 118.9 (d), 112.4 (s), 111.2 (d), 62.6 (t), 28.8 (t).}
\]

\[
\text{FTIR (cm}^{-1}\text{) 3540, 3398, 3316, 2933, 2858, 1456, 1339, 1043, 741.}
\]

\[
\text{EIMS } m/z \text{ (%) 161 (M}^+\text{, 27), 130 (100). CIMS } m/z \text{ (%) 162 (M}^+\text{ + 1, 100), 130 (54).}
\]

\[
\text{HREIMS } m/z \text{ calcd. for C}_{16}\text{H}_{11}\text{NO (161.0841), found 161.0841.}
\]
Indole-3-carboxylic acid (104)

\[
\text{COOH}
\]

\[
\text{NH}
\]

\[1^1\text{H NMR (300 MHz, DMSO-\text{d}_6)} \delta 11.94 \text{ (br s, 1H, D}_2\text{O exchangeable), 11.82 \text{ (br s, 1H, D}_2\text{O exchangeable), 8.01 (m, 2H), 7.47 (d, J = 8 Hz, 1H), 7.17 (m, 2H).}
\]

\[1^3\text{C NMR (75.5 MHz, DMSO-\text{d}_6)} \delta 166.0 \text{ (s), 136.4 (d), 132.3 (s), 126.0 (s), 122.1 (d), 121.0 (d), 120.6 (d), 112.2 (d), 107.4 (s).}
\]

FTIR (cm\(^{-1}\)) 3315, 2400-3500, 1665, 1459, 1241, 1107, 751.

EIMS \(m/z\) (%) 161 (M\(^{+}\), 21), 144 (17), 117 (100).

HREIMS \(m/z\) calcd. for C\(_9\)H\(_7\)NO\(_2\) (161.0477), found 161.0479.
4.3 Cyclobrassinin

4.3.1 Time-course experiments

4.3.1.1 Biotransformation of cyclobrassinin by Phoma lingam isolates

**Typical time-course procedure:** four 250 mL Erlenmeyer flasks each containing 100 mL of minimal medium were employed. Three of the flasks were inoculated with spores (2 \( \times \) 10⁶) of *P. lingam* isolate BJ-125 or Unity. All the flasks were incubated at 25 ± 2 °C on a shaker at 150 rpm. After 48 hours solutions of cyclobrassinin (2, final concentrations 2.1 \( \times \) 10⁻⁴ M and 4.2 \( \times \) 10⁻⁴ M) in DMSO [final concentration 0.5% (v/v)] were added to fungal cultures in two of the flasks and to uninoculated medium (control) in another flask. To fungal cultures (control) in the third flask 500 μL of DMSO was added. Samples (10 mL each) were taken from the flasks immediately after adding the compounds. Subsequently, 10 mL samples were taken after 3, 6, 9, 12, 24 hours, and final samples after 28 hours. The 10 mL samples were extracted with 20 mL of solvent, evaporated under reduced pressure and the residues analyzed by HPLC as described in section 4.2.1.1.

After 28 hours fungal cultures were filtered to separate mycelia from the broth. Each broth (40 mL) was extracted with diethyl ether (40 mL each) to give neutral, acidic and basic extracts. The contents of the uninoculated flask (40 mL) were similarly extracted to furnish neutral, acidic and basic extracts. The mycelium from each flask was air dried, ground to a powder and extracted with diethyl ether (2 \( \times \) 20 mL). Each extract was processed and analyzed by TLC and HPLC as described in section 4.2.1.1. Cyclobrassinin was stable in uninoculated media up to 14 days.
4.3.1.2 Biotransformation of cyclobrassinin by Rhizoctonia solani

Typical time-course procedure: four 250 mL Erlenmeyer flasks each containing 100 mL of potato dextrose broth medium were employed. Three of the flasks were inoculated with mycelia (5 × 5 mm diameter plug) of R. solani isolate AG2-1. All the flasks were incubated at 25 ± 2 °C on a shaker at 150 rpm. After 48 hours solutions of cyclobrassinin (2, final concentration $2.1 \times 10^{-4}$ M) in DMSO [final concentration 0.5% (v/v)] were added to fungal cultures in two of the flasks and to uninoculated medium (control) in another flask. To fungal cultures (control) in the third flask 500 μL of DMSO was added. Samples (5 mL each) were taken from the flasks immediately after adding the compounds. Subsequently 10 mL samples were taken after 6, 12 hours, 1, 2, 4, 6 days and final samples after 7 days. The 5 and 10 mL samples were extracted with 10 and 20 mL of solvent, respectively, evaporated under reduced pressure and the residues analyzed by HPLC as described in section 4.2.1.1.

After 7 days fungal cultures were filtered to separate mycelia from the broth. Each broth (35 mL) was extracted with diethyl ether (35 mL) to yield neutral, acidic and basic extracts as described in section 4.1. The contents (35 mL) of the uninoculated flask were similarly extracted to furnish neutral, acidic and basic extracts. The mycelium from each flask was air dried, ground to a powder and extracted with diethyl ether (2 × 20 mL). Each extract was processed and analyzed by TLC and HPLC as described in section 4.2.1.1. Cyclobrassinin was stable in uninoculated media up to 14 days.

4.3.2 Isolation of metabolites

4.3.2.1 Biotransformation of cyclobrassinin by virulent Phoma lingam isolate BJ-125

Typical scale up procedure: five 250 mL Erlenmeyer flasks each containing 100 mL of minimal medium were employed. All the flasks were inoculated with spores of
P. lingam isolate BJ-125. After 48 hours solutions of cyclobrexinin (2, final concentration \(4.0 \times 10^{-4} \text{ M}\)) in DMSO [final concentration 0.5% (v/v)] were added to fungal cultures in four flasks. To the fungal cultures (control) in the fifth flask 500 µL of DMSO was added. One test flask and the control flask were chosen for sampling. Samples (10 mL each) were taken after 6, 12, 24 hours and final samples after 30 hours. The 10 mL samples were extracted and analyzed as described in section 4.2.1.1.

The final samples from the 30-hour-old cultures were filtered to separate mycelium from the broth. The combined broth (370 mL) was extracted with diethyl ether (400 mL). The ethereal extract was dried (\(\text{Na}_2\text{SO}_4\)) and the solvent evaporated under reduced pressure. The residue was then analyzed by TLC and HPLC.

Preparative TLC [CH\(_2\)Cl\(_2\)-MeOH (95 : 5)] on the broth extract (39.6 mg) yielded dioxibrassinin (36, 7.6 mg).

4.3.2.2 Biotransformation of cyclobrexinin by avirulent Phoma lingam isolate Unity

Typical scale up procedure: five 250 mL Erlenmeyer flasks each containing 100 mL of minimal medium were employed. All the flasks were inoculated with spores of P. lingam isolate Unity. After 48 hours solutions of cyclobrexinin (2, final concentration \(4.0 \times 10^{-4} \text{ M}\)) in DMSO [final concentration 0.5% (v/v)] were added to fungal cultures in four flasks. To the fungal cultures (control) in the fifth flask 500 µL of DMSO was added. One test flask and the control flask were chosen for sampling. Samples (10 mL each) were taken after 9, 24 hours and final samples after 28 hours. The 10 mL samples were extracted and analyzed as described in section 4.2.1.1.

The final samples from the 28-hour-old cultures were filtered to separate mycelium from the broth. The combined broth (380 mL) was extracted with diethyl ether (400 mL). The ethereal extract was dried (\(\text{Na}_2\text{SO}_4\)) and the solvent evaporated under reduced pressure. The residue from the broth extract was analyzed by TLC and HPLC.
Preparative TLC [RP-8 plate, 20 cm x 20 cm, 0.25 mm, CH$_3$CN-H$_2$O (40 : 60)] on the broth extract (21.7 mg) yielded brassilixin (29, 2.6 mg), and 3-methylenaminoindole-2-thione (34, 2.4 mg).

4.3.2.3 Biotransformation of cyclobrassinin by Rhizoctonia solani AG2-1

**Typical scale up procedure:** four 250 mL Erlenmeyer flasks each containing 100 mL of potato dextrose medium were employed. All the flasks were inoculated with mycelia of *R. solani* AG2-1. After 48 hours solutions of cyclobrassinin (2, final concentration 2.0 x 10$^{-4}$ M) in DMSO [final concentration 0.5% (v / v)] were added to fungal cultures in three flasks. To the fungal cultures (control) in the fourth flask 500 µL of DMSO was added. One test flask and the control flask were chosen for sampling. Samples (5 mL each) were taken immediately after adding the compounds. Subsequently, 10 mL samples were taken after 6, 24 hours, 2, 4 days and final samples after 4 days. The 10 mL samples were extracted and analyzed as described in section 4.2.1.1.

The final samples from the 4-day-old cultures were filtered to separate mycelium from the broth. The combined broth (155 mL) was extracted with diethyl ether (155 mL). The ethereal extract was dried (Na$_2$SO$_4$) and the solvent evaporated under reduced pressure. The residue from the broth extract was analyzed by TLC and HPLC.

Preparative TLC [CH$_2$Cl$_2$-MeOH (97 : 3)] on the broth extract (11.6 mg) yielded 2-mercaptopindole-3-carboxaldehyde (32, 1.1 mg), brassicanal A (52, 1.1 mg) and 5-hydroxybrassicanal A (129, 2.5 mg).

4.3.3 Bioassays

4.3.3.1 Bioassays with virulent and avirulent Phoma lingam isolates

The bioassays were carried out on six- and twelve-well tissue culture plates as described in section 4.2.3.
4.3.3.2 *Bioassays with Rhizoctonia solani*

**Radial growth of mycelia:** regular size Petri dishes (100 mm × 15 mm) were employed for these bioassays. Solutions of the test compound (final concentrations $5 \times 10^{-4}$ M and $1 \times 10^{-4}$ M) in DMSO (final concentration 2%) were prepared by dissolving the amount of compound to give each concentration in DMSO and adding the solution to potato dextrose agar medium (15 mL) at ca. 50 °C in a Falcon tube. The agar medium and the compound were quickly mixed (vortex mixer) and poured into three Petri dishes (ca. 5 mL each). A control containing only DMSO and the potato dextrose agar was similarly poured into three Petri dishes. After preparation the agar plates were allowed to set for at least one hour before inoculation with mycelia of the fungus. Inoculation was done by placing a plug of mycelia at the center of each plate. The plates were then incubated under constant light at 25 ± 2 °C for seven days. The diameter of the circle covered by the mycelial mass was measured daily. Each bioassay was repeated at least three times.
4.3.4 Synthesis of compounds

Brassinin (1)

Carbon disulfide (330 µL, 5.5 mmol) was added to a mixture of indole-3-methanamine (21, 728 mg, 5.0 mmol), triethylamine (760 µL), and pyridine (4 mL). The mixture was kept stirred at 0 °C for one hour followed by addition of methyl iodide (340 µL, 5.5 mmol). The reaction mixture was kept at 3 °C (in the refrigerator) for 20 hours then poured into 1.5 M H₂SO₄ (50 mL) and extracted with diethyl ether (3 × 50 mL). The combined extracts were washed successively with water (150 mL), saturated NaHCO₃ (150 mL) and water (150 mL). The ether solution was dried over Na₂SO₄ and the solvent evaporated under reduced pressure to yield 864 mg (73%) of brassinin (1).
Cyclobrassinin (2)

Pyridinium bromide perbromide (640 mg, 2 mmol) was added in small portions to a solution of brassinin (1, 472 mg, 2 mmol) in dry THF (50 mL) at room temperature. The mixture was stirred at room temperature for 30 minutes, then basified with DBU (500 µL). After stirring for another hour at room temperature the solvent was evaporated and the residue separated by flash column chromatography (silica gel, CH₂Cl₂) to afford 204 mg (44%) of cyclobrassinin (2).

¹H NMR (300 MHz, CDCl₃) δ 7.92 (br s, 1H, D₂O exchangeable), 7.46-7.49 (m, 1H), 7.30-7.33 (m, 1H), 7.12-7.20 (m, 2H), 5.08 (s, 2H), 2.58 (s, 3H).

¹³C NMR (75.5 MHz, CDCl₃) δ 152.3 (s), 136.9 (s), 125.4 (s), 122.5 (s), 122.3 (d), 120.5 (d), 117.4 (d), 110.9 (d), 104.1 (s), 48.9 (t), 15.5 (q).

FTIR (cm⁻¹) 3373, 2922, 2832, 1601, 1449, 1429, 1338, 978, 914, 904, 744.

EI-MS m/z (%) 234 (M⁺, 29), 161 (100), 160 (23).
HREIMS $m/z$ calcd. for $C_{11}H_{10}N_2S_2$ (234.0285), found 234.0290.

**Indole-3-methanamine (21)**

![Diagram of chemical structures](image)

Methyl iodide (7.5 mL, 120 mmol) was added to a solution of gramine (23, 1026 mg, 6.0 mmol) in dry THF (60 mL) and stirred at room temperature for one hour. The solvent was evaporated under reduced pressure (rotovap) and concentrated ammonia solution (29%, 225 mL) added. The reaction mixture was then stirred at room temperature for two hours. A saturated solution of NaCl (225 mL) was added and the mixture extracted with $CH_2Cl_2$-MeOH (90: 10) (3 × 225 mL). The combined extracts were washed with saturated NaCl (300 mL) and dried over $Na_2SO_4$. Evaporation of the solvent yielded 904 mg of crude product. Purification by flash column chromatography [$CHCl_3$-MeOH-NH$_4$OH (80 : 20 : 1)] on silica gel gave 336 mg (47%) of indole-3-methanamine (21) and 177 mg of starting material (23). The % yield was calculated after subtraction of the recovered starting material.
Brassilexin (29)

Activated carbon (35 mg, 2.9 mmol) was added to a solution of 3-methylenaminoindole-2-thione (34, 51.0 mg, 0.29 mmol) in MeOH (2 mL). The reaction mixture was stirred at room temperature for 24 hours, filtered through a tight cotton plug, and the solvent evaporated under reduced pressure (rotovap) to yield 41 mg of brassilexin (29, 82% yield).

$^1$H NMR (500 MHz, CD$_3$CN) δ 9.81 (br s, 1H, D$_2$O exchangeable), 8.68 (s, 1H), 7.90 (d, J = 8 Hz, 1H), 7.55 (d, J = 8 Hz, 1H), 7.32 (dd, J = 8, 8 Hz, 1H), 7.22 (dd, J = 8, 8 Hz, 1H).

$^{13}$C NMR (125.8 MHz, CD$_3$OD) δ 160.5 (s), 148.7 (d), 146.0 (s), 128.4 (s), 124.9 (d), 121.5 (d), 121.4 (s), 120.9 (d), 112.9 (d).

FTIR (cm$^{-1}$) 3274, 3140, 3070, 2924, 2853, 1645, 1504, 1465, 1368, 1240, 856, 744.

EIMS $m/z$ (%) 174 (M$^+$, 100), 146 (15), 142 (16).

CIMS $m/z$ (%) 175 (M$^+$ + 1, 100), 145 (4).
HREIMS m/z calcd. for C₉H₆N₂S (174.0252), found 174.0253.

2-Indolinethione (31)

Phosphorous pentasulfide (0.313 g, 0.25 equiv) was added to a slurry of oxindole (50, 0.75 g, 5.6 mmol) and sea sand (0.75 g), in benzene (15 mL). The whole mixture was heated to reflux with stirring for 80 minutes. After cooling to room temperature the reaction mixture was filtered and the residue extracted with benzene (3 × 15 mL). The filtrate and the benzene extracts were combined and evaporated under reduced pressure. Flash column chromatography [CH₂Cl₂-MeOH (98 : 2)] on silica gel gave 422 mg (50%) of 2-indolinethione (31).

¹H NMR (300 MHz, CDCl₃) δ 10.47 (br s, 1H, D₂O exchangeable), 7.26 (m, 2H), 7.12 (dd, J = 8, 7 Hz, 1H), 7.00 (d, J = 8 Hz, 1H), 4.08 (s, 2H).

¹³C NMR (75.5 MHz, CDCl₃) δ 203.9 (s), 144.4 (s), 130.6 (s), 128.3 (d), 124.5 (d), 124.3 (d), 110.2 (d), 49.4 (t).

FTIR (cm⁻¹) 3145, 1621, 1508, 1467, 1451, 1338, 1114, 746.
EIMS $m/z$ (%) 149 ($M^+$, 100), 121 (15), 117 (32).

CIMS $m/z$ (%) 150 ($M^+ + 1$, 100), 118 (8).

HREIMS $m/z$ calcld. for $C_9H_7NS$ (149.0299), found 149.0298.

**2-Mercaptoindole-3-carboxaldehyde (32)**

![Chemical Structure](image)

Hexane washed NaH (60%, 745 mg, 0.02 mol) was added slowly to a solution of 2-indolinethione (31, 250 mg, 1.7 mmol) in HCOOEt (5 mL, 0.06 mol). After adding NaH the reaction mixture set to a thick paste and was left overnight (12 hours) at room temperature. Water (10 mL) was slowly added (dropwise), the mixture acidified with 2.5 M HCl, and the precipitate that formed was filtered and washed with water. The precipitate was dried and yielded 106 mg of 2-mercaptoindole-3-carboxaldehyde. The remaining aqueous layer was then extracted with EtOAc ($2 \times 40$ mL), the combined extracts dried over Na$_2$SO$_4$ and the solvent evaporated to yield 168 mg of 2-mercaptoindole-3-carboxaldehyde (32). Total yield of 2-mercaptoindole-3-carboxaldehyde was 274 mg (92%).
$^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 9.37 (s, 1H), 8.15 (d, J = 8 Hz, 1H), 7.48 (d, J = 8 Hz, 1H), 7.37 (dd J = 8, 8 Hz, 1H), 7.27 (dd, J = 8, 8 Hz, 1H).

$^1$H NMR (300 MHz, CD$_3$CN) $\delta$ 10.58 (br s, 1H, D$_2$O exchangeable), 9.65 (s, 1H), 8.12 (d, J = 8 Hz, 1H), 7.52 (d, J = 8 Hz, 1H), 7.37 (dd, J = 8, 8 Hz, 1H), 7.29 (dd, J = 8, 8 Hz, 1H).

$^{13}$C NMR (75.5 MHz, CD$_3$OD) $\delta$ 187.1 (d), 139.1 (s), 136.1 (s), 126.9 (d), 126.6 (s), 124.5 (d), 122.7, (d) 122.3 (s), 113.2 (d).

FTIR (cm$^{-1}$) 3229, 3052, 1621, 1454, 1341, 1247, 1216, 742.

EIMS m/z (%) 177 (M$^+$, 100), 149 (15), 148 (23), 121 (11), 117 (11).

CIMS m/z (%) 178 (M$^+$ + 1, 100), 164 (12), 150 (10), 146 (11).

HREIMS m/z calcd. for C$_9$H$_7$NOS (177.0248), found 177.0247.
2-Mercaptoindole-3-carboxaldehyde oxime (33)

To a solution of 2-mercaptoindole-3-carboxaldehyde (32, 100 mg, 0.56 mmol) in ethanol (2 mL) was added 1 mL of 1 M NH₂OH (1.12 mmol, 2 equiv) and the mixture refluxed for 1 hour. Water (10 mL) was added to the reaction mixture and the resulting precipitate filtered and dried to yield 47.6 mg of 2-mercaptoindole-3-carboxaldehyde oxime. The filtrate was extracted with EtOAc (2 × 20 mL), the combined EtOAc extracts dried over Na₂SO₄, and the solvent evaporated under reduced pressure to furnish another 65.8 mg of 2-mercaptoindole-3-carboxaldehyde oxime. Overall yield of the oxime (33) was 113.4 mg (100%).

¹H NMR (300 MHz, CD₃CN) δ 9.92 (br s, 1H, D₂O exchangeable), 8.52 (s, 1H, D₂O exchangeable), 8.04 (d, J = 8 Hz, 1H), 7.95 (s, 1H), 7.42 (d, J = 8 Hz, 1H), 7.31 (dd, J = 8, 7 Hz, 1H), 7.18 (dd, J = 8 Hz, 7 Hz, 1H).

¹³C NMR (75.5 MHz, CD₃CN) δ 145.2 (d), 138.9 (s), 131.1 (s), 126.0 (d), 125.7 (s), 123.5 (d), 122.4 (d), 117.2 (s), 112.6 (d).

FTIR (cm⁻¹) 3383, 3323, 1618, 1405, 1340, 963, 902, 844, 744.
EIMS m/z (%) 174 (M⁺ - 18, 100), 149 (51).

HREIMS m/z calcd. for C₇H₈N₂S (C₇H₈N₂OS·H₂O, 174.0252), found 174.0251.

3-Methylenaminoindole-2-thione (34)

Titanium trichloride (103 mg, 0.66 mmol, 2 equiv) was added in small portions to a solution of 2-mercaptoindole-3-carboxaldehyde oxime (33, 64 mg, 0.33 mmol), NaBH₃CN (63 mg, 1 mmol) and NH₄OAc (32 mg, 0.4 mmol) in methanol (3 mL). The mixture was stirred at room temperature for 30 minutes, followed by addition of water (20 mL) and extraction with diethyl ether (3 × 20 mL). The combined ether extracts were washed with water (120 mL), dried over Na₂SO₄ and the solvent evaporated under reduced pressure to yield 50.1 mg of 3-methylenaminoindole-2-thione (34, 85%).

¹H NMR (500 MHz, CD₂Cl₂) δ 11.13 (br s, 1H, D₂O exchangeable), 8.98 (br s, 1H, D₂O exchangeable), 8.15 (dd, J = 15, 8 Hz, 1H), 7.41 (d, J = 7.5 Hz, 1H), 7.11 (m, 3H), 6.25 (br s, 1H, D₂O exchangeable).

153
$^1$H NMR (500 MHz, CD$_3$CN) $\delta$ 11.00 (br s, 1H, D$_2$O exchangeable), 9.81 (br s, 1H, D$_2$O exchangeable), 8.25 (dd, J = 15.0, 8 Hz, 1H), 7.45 (d, J = 7.5 Hz, 1H), 7.28 (br s, 1H, D$_2$O exchangeable), 7.10 (m, 3H).

$^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 8.30 (s, 1H), 7.45 (d, J = 7 Hz, 1H), 7.05 (m, 3H).

$^{13}$C NMR (125.8 MHz, CD$_2$Cl$_2$) $\delta$ 179.0 (s), 148.9 (d), 138.0 (s), 128.3 (s), 124.1 (d), 122.2 (d), 115.3 (d), 109.8 (d), 108.4 (s).

$^{13}$C NMR (125.8 MHz, CD$_3$CN) $\delta$ 179.0, 151.4, 151.2, 139.0, 129.4, 124.3, 122.4, 116.2, 115.9, 110.4, 108.2.

$^{13}$C NMR (125.8 MHz, CD$_3$OD) $\delta$ 177.6, 153.7, 151.7, 148.7, 140.4, 139.5, 129.9, 126.9, 125.0, 124.9, 124.1, 123.4, 122.5, 121.6, 120.9, 119.6, 115.8, 112.9, 111.7, 110.8, 110.5, 108.6.

FTIR (cm$^{-1}$) 3238, 3128, 3067, 2924, 2852, 1641, 1623, 1549, 1446, 1398, 1303, 1233, 1210, 739.

EIMS $m/z$ (%) 176 (M$^+$, 100), 149 (46), 117 (11).

CIMS $m/z$ (%) 177 (M$^+$ + 1, 100), 164 (11), 145 (13).

HREIMS $m/z$ calcd. for C$_9$H$_8$N$_2$S (176.0408), found 176.0412.

Acetylation of 3-methylenaminooindole-2-thione (34): acetic anhydride (ca. 100 µL) and pyridine (ca. 20 µL) were added to a solution of 34 (4 mg) in CH$_3$CN (500 µL). The
reaction mixture was stirred at room temperature for 30 minutes and the solvent evaporated under reduced pressure. The residue was dissolved in toluene (1 mL) and the solvent again evaporated under reduced pressure. Preparative TLC (CH$_2$Cl$_2$) resulted in the isolation of acetyl 3-methylenaminoindole-2-thione (131, 1.8 mg) and diacetyl 3-methylenaminoindole-2-thione (132, 3.1 mg).

**Acetyl-3-methylenaminoindole-2-thione (131)**

![NMR Structure of 131](image)

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 13.14 (br s, 1H, D$_2$O exchangeable) 9.21 (br s, 1H, D$_2$O exchangeable), 8.48 (d, $J$ = 11.5 Hz, 1H), 7.51 (d, $J$ = 7.5 Hz, 1H), 7.22 (dd, $J$ = 7.5, 7.5 Hz, 1H), 7.14 (dd, $J$ = 7.5, 7.5 Hz, 1H), 7.03 (d, $J$ = 7.5 Hz, 1H), 2.32 (s, 3H).

$^{13}$C NMR (125.8 MHz, CDCl$_3$) $\delta$ 184.8 (s), 169.4 (s), 139.4 (s), 135.1 (d), 126.9 (d), 126.5 (s), 123.2 (d), 117.8 (d), 114.2 (s), 109.8 (d), 23.9 (q).

FTIR (cm$^{-1}$) 3187, 1706, 1618, 1434, 1256, 744.

EIMS $m/z$ (%) 218 (M$^+$, 88), 176 (100), 149 (60).
CIMS $m/z$ (%) 219 ($M^+ + 1$, 55), 189 (37), 164 (26), 132 (50).

HREIMS $m/z$ calcd. for C$_{11}$H$_{10}$N$_2$OS (218.0514), found 218.0515.

**Diacetyl-3-methylenaminondole-2-thione (132)**

![Chemical Structure](attachment:image.png)

$^1$H NMR (500 MHz, CD$_2$Cl$_2$) $\delta$ 13.40 (br s, 1H, D$_2$O exchangeable), 8.57 (d, $J = 11.5$ Hz, 1H), 7.98 (dd, $J = 7$, 1 Hz, 1H), 7.56 (dd, $J = 7$, 1 Hz, 1H), 7.28 (m, 3H), 2.97 (s, 3H), 2.33 (s, 3H).

$^{13}$C NMR (125.8 MHz, CD$_2$Cl$_2$) $\delta$ 194.7 (s), 179.5 (s), 175.8 (s), 144.6 (s), 140.7 (d), 131.2 (d), 130.4 (s), 129.0 (d), 120.3 (d), 119.0 (s), 118.9 (d), 28.3 (q), 23.0 (q).

FTIR (cm$^{-1}$) 2924, 2852, 1725, 1705, 1617, 1599, 1364, 1290, 1267, 1168, 968, 760, 744.

EIMS $m/z$ (%) 260 ($M^+$, 38), 218 (44, 176 (100), 149 (52).
HREIMS $m/z$ calcd. for $C_{13}H_{12}N_2O_2S$ (260.0620), found 260.0624.

Hydrolysis of 3-methylenaminindoole-2-thione (34): trifluoroacetic acid (200 µL) and $H_2O$ (ca. 20 µL) were added to a solution of 34 (35 mg) in $CH_3CN$ (1 mL). The reaction mixture was kept at room temperature for 120 hours. Evaporation of the solvent under reduced pressure followed by preparative TLC [EtOAc-Hex (40 : 60)] yielded 2-mercaptopindoole-3-carboxaldehyde (32, 20 mg) and brassilexin (29, 0.2 mg).

**Brassicanal A (52)**

![Diagram of molecular structure](image)

A solution of diazomethane (excess) in ether was added to a solution of 2-mercaptopindoole-3-carboxaldehyde (32, 20.3 mg, 0.11 mmol) in ether (1 mL), and the reaction mixture kept at room temperature for 4 hours. The solvent was then allowed to evaporate overnight in the fumehood. This afforded 21.9 mg of brassicanal A (52, 100% yield).

$^1$H NMR (300 MHz, $CD_3CN$) $\delta$ 10.14 (br s, 1H, $D_2O$ exchangeable), 10.14 (s, 1H), 8.05 (m, 1H), 7.45 (m, 1H), 7.23 (m, 2H), 2.64 (s, 3H).
$^{13}$C NMR (75.5 MHz, CD$_3$CN) $\delta$ 184.7 (d), 146.6 (s), 137.9 (s), 127.2 (s), 124.4 (d), 123.6 (d), 120.5 (d), 116.8 (s), 112.2 (d), 16.9 (q).

FTIR (cm$^{-1}$) 3152, 3054, 2940, 1627, 1581, 1450, 1354, 1229, 847, 753.

EIMS $m/z$ (%): 191 (M$^+$, 100), 176 (23), 158 (55), 148 (13).

CIMS $m/z$ (%): 192 (M$^+$ + 1, 100), 146 (7).

HREIMS $m/z$ calcd. for C$_{10}$H$_8$NOS (191.0405), found 191.0402.

3-Hydroxymethyleneoxindole (134)

A solution of NaOEt (500 $\mu$L) prepared from 62 mg of sodium and 1 mL of absolute ethanol was added to a slurry of oxindole (133 mg, 1 mmol) in HCOOEt (500 $\mu$L). The reaction mixture was stirred at room temperature for 1 hour followed by neutralization with 2.5 M HCl. The resulting precipitate was filtered and dried to afford 3-hydroxymethyleneoxindole (134, 130 mg, 81%).
$^1$H NMR (300 MHz, CD$_3$OD) δ 7.90 (s, 1H), 7.63 (d, J = 7.5 Hz, 1H), 7.09 (dd, J = 7.5, 7.5 Hz, 1H), 6.99 (dd, J = 7.5, 7.5 Hz, 1H).

$^{13}$C NMR (75.5 MHz, CD$_3$OD) δ 174.0 (s), 156.6 (d), 139.7 (s), 127.5 (d), 124.3 (s), 123.8 (d), 122.9 (d), 110.4 (d), 108.4 (s).

FTIR (cm$^{-1}$) 3157, 2400-3500, 1689, 1619, 1464, 1195, 1006, 778, 749.

CIMS $m/z$ (%) 162 (M$^+$ + 1, 100).

EIMS $m/z$ (%) 161 (M$^+$, 100), 144 (27), 133 (20).

HREIMS $m/z$ calcd. for C$_9$H$_7$NO$_2$ (161.0477), found 161.0477.
3-Methoxymethyleneoxindole (137)

Ammonium chloride (13.3 mg, 0.25 mmol) was added to a solution of 3-hydroxymethyleneoxindole (134, 80 mg, 0.5 mmol) in methanol (500 µL) and the reaction mixture refluxed for 1 hour. The product crystallized out on cooling to room temperature. Filtration and drying of the crystals afforded 3-methoxymethyleneoxindole (137, 62 mg, 71%).

$^1$H NMR (300 MHz, CDCl$_3$) δ 8.69 (br s, 1H, D$_2$O exchangeable), 7.58 (d, J = 7.5 Hz, 1H), 7.55 (s, 1H), 7.15 (dd, J = 7.5, 7.5 Hz, 1H), 7.00 (dd, J = 7.5, 7.5 Hz, 1H), 6.88 (d, J = 7.5 Hz, 1H), 4.10 (s, 3H).

$^{13}$C NMR (75.5 MHz, CDCl$_3$) δ 171.7 (s), 158.2 (d), 138.7 (s), 127.5 (d), 123.2 (d), 122.3 (s), 122.2 (d), 109.9 (d), 108.5 (s), 63.4 (q).

FTIR (cm$^{-1}$) 3159, 1709, 1683, 1649, 1464, 1274, 1207, 1141, 950, 734.

EIMS m/z (%) 175 (M$^+$, 100), 146 (50), 144 (76), 132 (24).
CIMS $m / z$ (%) 176 ($M^+ + 1, 100$).

HREIMS $m / z$ calcd. for C$_{10}$H$_8$NO$_2$ (175.0633), found 175.0632.

3-Chloromethyleneoxindole (135)

![Chemical structure](image)

Thionyl chloride (500 µL) was added to 3-hydroxymethyleneoxindole (134, 45 mg, 0.28 mmol) and the solution kept at room temperature (25 °C) for 1 hour. The product precipitated out and excess SOCl$_2$ was removed by evaporation under reduced pressure to afford 3-chloromethyleneoxindole (135, 46.5, 92%).

$^1$H NMR (500 MHz, CDCl$_3$) δ 8.60 (br s, 1H, D$_2$O exchangeable), 7.95 (d, J = 7.5 Hz, 1H), 7.45 (s, 1H), 7.27 (dd, J = 7.5, 7.5 Hz, 1H), 7.05 (dd, J = 7.5, 7.5 Hz, 1H), 6.88 (d, J = 7.5 Hz, 1H).

$^{13}$C NMR (125.8 MHz, CDCl$_3$) δ 167.8 (s), 141.1 (s), 130.5 (d), 129.7 (d), 129.6 (s), 124.9 (d), 122.5 (d), 121.1 (s), 110.1 (d).

FTIR (cm$^{-1}$) 3185, 1716, 1621, 1464, 746.
EIMS \(m/z\) (%) 179 (M⁺, 92), 144 (100), 130 (39).

HREIMS \(m/z\) calcd. for \(C_9H_8NOCl\) (179.0138), found 179.0139.

4.3.5 Spectroscopic Data

Dioxibrassinin (36)

\[
\text{\text{[\alpha]}_D +21.3 \text{ (MeOH; c 0.0024).}}
\]

\(1^1\text{H NMR (300 MHz, CD}_3\text{OD)} \delta 7.36 \text{ (d, } J = 7.5 \text{ Hz, 1H), 7.27 (dd, } J = 7.5, 7.5 \text{ Hz, 1H), 7.01 (dd, } J = 7.5, 7.5 \text{ Hz, 1H), 6.89 (d, } J = 7.5 \text{ Hz, 1H), 4.32 (d, } J = 14 \text{ Hz, 1H), 3.98 (d, } J = 14 \text{ Hz, 1H), 2.53 (s, 3H).}
\]

\(13^1\text{C NMR (125.8 MHz, CD}_3\text{OD)} \delta 201.9 \text{ (s), 180.6 (s), 142.7 (s), 131.1 (2C, d, s), 125.9 (d), 123.9 (d), 111.4 (d), 76.3 (s), 53.3 (t), 18.2 (q).}
\]

FTIR (cm⁻¹) 3273, 3223, 2918, 2850, 1720, 1622, 1470, 1378, 1189, 754.
EIMS $m/z$ (\%) 268 ($M^+$, 12), 220 (25), 191 (15), 164 (29), 149 (100).

CIMS $m/z$ (\%) 269 ($M^+ + 1$, 11), 238 (22), 163 (100), 146 (88).

HREIMS $m/z$ calcd. for C$_{11}$H$_{12}$N$_2$O$_2$S$_2$ (268.0340), found 268.0345.

5-Hydroxybrassicanal A (129)

\[ \text{HO} \begin{array}{c} \text{CHO} \\ \text{SCH}_3 \end{array} \]

1H NMR (500 MHz, CD$_3$OD) $\delta$ 9.96 (s, 1H), 7.48 (d, $J = 2.5$ Hz, 1H), 7.21 (d, $J = 8.5$ Hz, 1H), 6.74 (dd, $J = 8.5$, 2.5 Hz, 1H), 2.64 (s, 3H).

1H NMR (500 MHz, CD$_3$CN) $\delta$ 10.07 (s, 1H), 9.97 (br s, 1H, D$_2$O exchangeable), 7.45 (d, $J = 2.5$ Hz, 1H), 7.26 (d, $J = 9.0$ Hz, 1H), 6.74 (dd, $J = 9.0$, 2.5 Hz, 1H), 6.69 (s, 1H, D$_2$O exchangeable), 2.61 (s, 3H).

13C NMR (125.8 MHz, CD$_3$OD) $\delta$ 185.7 (d), 154.9 (s), 148.1 (s), 133.0 (s), 128.3 (s), 117.3 (s), 114.1 (d), 112.7 (d), 106.0 (d), 17.0 (q).
FTIR (cm⁻¹) 3171, 2919, 2852, 2783, 2615, 1612, 1585, 1444, 1365, 1224, 861.

EIMS m/z (%) 207 (M⁺, 100), 192 (20), 174 (81), 164 (15).

CIMS m/z (%) 208 (M⁺ + 1, 100), 148 (12).

HREIMS m/z calcd. for C₁₀H₇NO₂S (207.0354), found 207.0353.

4.4 Other phytoalexin analogs

4.4.1 Time-course experiments

Typical time-course procedure described in section 4.2.1 was followed for biotransformations of methyl tryptamine carbamate and N₆-carbomethoxyindole-3-methanamine with virulent (BJ-125) and avirulent (Unity) *P. lingam* isolates. For biotransformations of cyclobrassinin homologue, the following time-course procedures were followed.

4.4.1.1 Biotransformation of cyclobrassinin homologue by virulent *Phoma lingam*

Typical time-course procedure: four 250 mL Erlenmeyer flasks each containing 100 mL of minimal medium were employed. Three of the flasks were inoculated with spores of *P. lingam* isolate BJ-125. After 48 hours solutions of cyclobrassinin homologue (6, final concentration 2 × 10⁻⁴ M) in DMSO [final concentration 0.5% (v/v)] were added to fungal cultures in two of the flasks and to uninoculated medium (control) in another flask. To fungal cultures (control) in the third flask 500 μL of DMSO was added. Samples (5 mL each) were taken from the flasks immediately after adding the compounds. Subsequently, 10 mL samples were taken after 1, 2, and final samples after 3 days. The 5 and 10 mL samples were extracted with 10 and
20 mL of solvent, respectively, evaporated under reduced pressure and the residues analyzed by HPLC as described in section 4.2.1.1.

After 3 days fungal cultures were filtered to separate mycelium from the broth. Each broth (75 mL) was extracted with diethyl ether (75 mL each) to give neutral, acidic and basic extracts as described in section 4.1. The contents of the uninoculated flask (75 mL) were similarly extracted to furnish neutral, acidic and basic extracts. The mycelium from each flask was air dried, ground to a powder and extracted with diethyl ether (2 × 20 mL). Each of the extracts was processed and analyzed by TLC and HPLC as described in section 4.2.1.1.

4.4.1.2 Biotransformation of cyclobrassinin homologue by avirulent Phoma lingam

Typical time-course procedure: four 250 mL Erlenmeyer flasks each containing 100 mL of minimal medium were set up as described in section 4.4.1.1. Samples (5 mL each) were taken from the flasks immediately after adding the compounds. Subsequently 10 mL samples were taken after 1, 2, 3, 4 days and final samples after 5 days. The 5 and 10 mL samples were extracted with 10 and 20 mL of solvent, respectively, evaporated under reduced pressure and the residues analyzed by HPLC as described in section 4.2.1.1.

After 5 days fungal cultures were filtered to separate mycelium from the broth. Each broth (55 mL) was extracted with diethyl ether (55 mL each) to give neutral, acidic and basic extracts as described in section 4.1. The contents of the uninoculated flask (55 mL) were similarly extracted to furnish neutral, acidic and basic extracts. The mycelium from each flask was air dried, ground to a powder and extracted with diethyl ether (2 × 20 mL). Each of the extracts was processed and analyzed by TLC and HPLC as described in section 4.2.1.1.
4.4.2 Isolation of metabolites

4.4.2.1 Biotransformation of methyl tryptamine carbamate by virulent Phoma lingam

Typical scale up procedure: ten 250 mL Erlenmeyer flasks each containing 100 mL of minimal medium were employed. Nine of the flasks were inoculated with spores of P. lingam isolate BJ-125. After 48 hours solutions of methyl tryptamine carbamate (4, final concentration $2.0 \times 10^{-4} \text{M}$) in DMSO [final concentration 0.5% (v/v)] were added to fungal cultures in eight flasks and to uninoculated medium (control) in another flask. To the fungal cultures (control) in the ninth flask 500 µL of DMSO was added. One test flask and the control flask were chosen for sampling. Samples (10 mL each) were taken after 0, 2, 6, 9, 13 days and final samples after 14 days. The 10 mL samples were extracted and analyzed as described in section 4.2.1.1.

The final samples from the 14-day-old cultures were filtered to separate mycelium from the broth. The combined broth (750 mL) was extracted with an equivalent amount of solvent to furnish neutral and acidic extracts as described in section 4.1. The ethereal extracts were dried ($\text{Na}_2\text{SO}_4$), the solvent evaporated under reduced pressure and the residues analyzed by TLC and HPLC.

Preparative TLC [CH$_2$Cl$_2$-MeOH (95 : 5)] on the broth neutral extract (96.6 mg) resulted mainly in isolation of recovered methyl tryptamine carbamate (4, 38 mg), phytotoxins and undetermined UV-inactive components. Preparative TLC with the same solvent system on the broth acidic extract (106.2 mg) yielded indole-3-acetic acid (117, ca. 2 mg), phytotoxins together with undetermined UV-inactive components.
4.4.2.2 Biotransformation of methyl tryptamine carbamate by avirulent Phoma lingam

Typical scale up procedure: eight 250 mL Erlenmeyer flasks each containing 100 mL of minimal medium were employed. Seven of the flasks were inoculated with spores of *P. lingam* isolate Unity. After 48 hours solutions of methyl tryptamine carbamate (4, final concentration $2.0 \times 10^{-4}$ M) in DMSO [final concentration 0.5% (v/v)] were added to fungal cultures in six flasks and to uninoculated medium (control) in another flask. To the fungal cultures (control) in the seventh flask 500 µL of DMSO was added. One test flask and the control flask were chosen for sampling. Samples (10 mL each) were taken after 0, 2, 6, 10 and final samples after 14 days. The 10 mL samples were extracted and analyzed as described in section 4.2.1.1.

The final samples from the 14-day-old cultures were filtered to separate mycelium from the broth. The combined broth (560 mL) was extracted with an equivalent amount of solvent to furnish neutral and acidic extracts as described in section 4.1. The ethereal extracts were dried (Na$_2$SO$_4$), the solvent evaporated under reduced pressure and the residues analyzed by TLC and HPLC.

Preparative TLC [CH$_2$Cl$_2$-MeOH (95 : 5)] on the broth neutral extract (17.8 mg) resulted in isolation of recovered methyl tryptamine carbamate (4, 1.5 mg), tryptophol (121, 1.5 mg), indole-3-carboxylic acid (104, 4.3 mg) together with unidentified UV-inactive components. The HPLC chromatograms of the acidic extract did not show any peaks of possible metabolites and was not pursued further.

4.4.2.3 Biotransformation of cyclobrassinin homologue by virulent Phoma lingam

Typical scale up procedure: four 250 mL Erlenmeyer flasks each containing 100 mL of minimal medium were employed. All the flasks were inoculated with spores of *P. lingam* isolate BJ-125. After 48 hours solutions of cyclobrassinin homologue (6, final
concentration \(2.0 \times 10^{-4} \text{ M}\) in DMSO [final concentration 0.5\% (v / v)] were added to fungal cultures in three flasks. To the fungal cultures (control) in the fourth flask 500 \(\mu\text{L}\) of DMSO was added. One test flask and the control flask were chosen for sampling. Samples (5 \(\text{mL}\) each) were taken immediately after adding the compounds. Subsequently 10 \(\text{mL}\) samples were taken after 6, 12, 24 hours and final samples after 24 hours. The 10 \(\text{mL}\) samples were extracted and analyzed as described in section 4.2.1.1.

The final samples from the 24-hour-old cultures were filtered to separate mycelium from the broth. The combined broth (365 \(\text{mL}\)) was extracted with diethyl ether (365 \(\text{mL}\)). The ethereal extract was dried (\(\text{Na}_2\text{SO}_4\)) and the solvent evaporated under reduced pressure. The residue from the broth extract was analyzed by TLC and HPLC.

Preparative TLC [EtOAc-Hex (40 : 60)] on the broth extract (19.8 mg) yielded cyclobrassinin homologue metabolite (142, 6.9 mg).

4.4.2.4 Biotransformation of cyclobrassinin homologue by avirulent Phoma lingam

**Typical scale up procedure**: six 250 \(\text{mL}\) Erlenmeyer flasks each containing 100 \(\text{mL}\) of minimal medium were employed. All the flasks were inoculated with spores of *P. lingam* isolate Unity. After 48 hours solutions of cyclobrassinin homologue (6, final concentration \(2.0 \times 10^{-4} \text{ M}\) in DMSO [final concentration 0.5\% (v / v)] were added to fungal cultures in five flasks. To the fungal cultures (control) in the sixth flask 500 \(\mu\text{L}\) of DMSO was added. One test flask and the control flask were chosen for sampling. Samples (5 \(\text{mL}\) each) were taken immediately after adding the compounds. Subsequently 10 \(\text{mL}\) samples were taken after 6 hours, 2, 4 days and final samples after 4 days. The 10 \(\text{mL}\) samples were extracted and analyzed as described in section 4.2.1.1.

The final samples from the 24-hour-old cultures were filtered to separate mycelium from the broth. The combined broth (465 \(\text{mL}\)) was extracted with diethyl ether (465 \(\text{mL}\)).
The ethereal extract was dried (Na$_2$SO$_4$) and the solvent evaporated under reduced pressure. The residue from the broth extract was analyzed by TLC and HPLC.

Preparative TLC [CH$_2$Cl$_2$-MeOH (97 : 3)] on the broth extract (28.5 mg) yielded cyclobrassinin homologue metabolite (142, 13.8 mg).

4.4.3 Bioassays

The antifungal activity of methyl tryptamine carbamate (4), N$_b$-carbomethoxyindole-3-methanamine (5), cyclobrassinin homologue (6) and their metabolites against P. lingam isolates was determined by the spore germination and radial growth of mycelia on V-8 juice agar as described in section 4.2.3.
4.4.4 Synthesis of compounds

Methyl tryptamine carbamate (4)

\[ \text{112} \xrightarrow{\text{NH}_2} \text{4} \]

A solution of N,O-bis(trimethylsilyl)acetamide (1.5 mmol) in CH\(_2\)Cl\(_2\) (1.25 mL) was added to a solution of tryptamine (112, 160 mg, 1.0 mmol) in CH\(_2\)Cl\(_2\) (6 mL) under argon and the mixture stirred at 20 °C for 1 hour. The reaction mixture was cooled to 0 °C, and a solution of methyl chloroformate (1.5 mmol) in CH\(_2\)Cl\(_2\) (1.25 mL) added. The reaction mixture was allowed to warm up to 20 °C over 1 hour, stirred at 20 °C for 1 h, cooled to 0 °C, quenched with pH 7 buffer (10 mL), and extracted with diethyl ether (2 × 20 mL). The ether extract was washed with brine (40 mL), dried (Na\(_2\)SO\(_4\)), filtered, and evaporated under reduced pressure to afford 187 mg (86%) of methyl tryptamine carbamate (4).

\(^1\text{H NMR (300 MHz, CDCl}_3\text{)} \delta \text{8.13 (br s, 1H, D}_2\text{O exchangeable), 7.61 (d, J = 8 Hz, 1H), 7.38 (d, J = 8 Hz, 1H), 7.23 (dd, J = 8, 8 Hz, 1H), 7.14 (dd, J = 8, 8 Hz, 1H), 7.03 (s, 1H), 4.78 (br s, 1H, D}_2\text{O exchangeable), 3.67 (s, 3H), 3.53 (m, 2H), 2.98 (m, 2H).}\

\(^13\text{C NMR (75.5 MHz, CDCl}_3\text{)} \delta \text{157.1 (s), 136.5 (s), 127.3 (s), 122.2 (d), 122.1 (d), 119.5 (d), 118.7 (d), 112.9 (s), 111.2 (d), 52.0 (q), 41.4 (t), 25.8 (t).} \]
FTIR (cm⁻¹) 3409, 3322, 2945, 1700, 1524, 1457, 1261, 743.

EIMS m/z (%) 218 (M⁺, 29), 143 (38), 130 (100).

CIMS m/z (%) 219 (M⁺ + 1, 100), 187 (46), 143 (28), 129 (67).

HREIMS m/z calcd. for 218.1055 (C₁₂H₁₄N₂O₂), found 218.1054.

**N₆-carbomethoxyindole-3-methanamine (5)**

![Chemical Structure](image)

A solution of N,O-bis(trimethylsilyl)acetamide (3.5 mmol) in CH₂Cl₂ (3 mL) was added to a solution of indole-3-methanamine (21, 415 mg, 2.84 mmol) in CH₂Cl₂ (10 mL) under argon and the solution stirred at 20 °C for 1 hour. The reaction mixture was cooled to 0 °C, and a solution of methyl chloroformate (3.5 mmol) in CH₂Cl₂ (3.0 mL) added. The reaction mixture was allowed to warm up to 20 °C over 1 hour, stirred at 20 °C for 1 hour, cooled to 0 °C, quenched with pH 7 buffer (20 mL), and extracted with diethyl ether (2 × 40 mL). The ether extract was washed with brine (80 mL), dried (Na₂SO₄), filtered, and evaporated under reduced pressure to give 430 mg (74%) of N₆-carbomethoxyindole-3-methanamine (5).
$^1$H NMR (300 MHz, CDCl$_3$) δ 8.13 (br s, 1H, D$_2$O exchangeable), 7.66 (dd, J = 8, 1 Hz, 1H), 7.39 (dd, J = 8, 1 Hz, 1H), 7.23 (ddd, J = 8, 8, 1 Hz, 1H), 7.13-7.18 (m, 2H), 4.88 (br s, 1H, D$_2$O exchangeable), 4.56 (d, J = 5 Hz, 2H), 3.70 (s, 3H).

$^{13}$C NMR (75.5 MHz, CDCl$_3$), 157.1 (s), 136.5 (s), 126.5 (s), 123.0 (d), 122.4 (d), 119.8 (d), 118.8 (d), 113.1 (s), 111.4 (d), 52.2 (q), 36.8 (t).

FTIR (cm$^{-1}$) 3407, 3322, 2950, 2870, 1699, 1521, 1457, 1258, 1236, 744.

EIMS m/z (%) 204 (M$^+$, 100), 189 (36), 171 (10), 145 (18), 130 (83).

CIMS m/z (%) 205 (M$^+$ + 1, 100), 189 (5), 145 (7), 130 (57).

HREIMS m/z calcd. for C$_{11}$H$_{12}$N$_2$O$_2$ (204.0899), found 204.0895.
Cyclobrassinin homologue (6)

Pyridinium bromide perbromide (400 mg, 1.25 mmol) was added in small portions to a solution of methyl tryptamine dithiocarbamate (3, 250 mg, 1 mmol) in CH$_2$Cl$_2$ (24 mL) at 25 °C. The reaction mixture was stirred at 25 °C for 20 min, then basified with DBU (400 μL) and refluxed for 10 minutes. The solvent was evaporated under reduced pressure and the mixture separated on a column (silica gel, CH$_2$Cl$_2$) to afford 159 mg (64%) of cyclobrassinin homologue (6).

$^1$H NMR (300 MHz, CDCl$_3$) δ 7.84 (br s, 1H), 7.39 (d, J = 8 Hz, 1H), 7.25 (d, J = 8 Hz, 1H), 7.16 (ddd, J = 8 Hz, 8 Hz, 1 Hz, 1H), 7.09 (ddd, J = 8 Hz, 8 Hz, 1 Hz, 1H), 4.34 (t, J = 6 Hz, 2H), 3.14 (t, J = 6 Hz, 2H), 2.47 (s, 3H).

$^{13}$C NMR (75.5 MHz, CDCl$_3$) δ 135.8 (s), 135.1 (s), 128.9 (s), 122.8 (d), 120.1 (d), 119.2 (s), 117.8 (d), 113.5 (s), 110.4 (d), 51.9 (t), 23.9 (t), 16.4 (q).

FTIR (cm$^{-1}$) 3388, 2947, 2893, 1591, 1584, 1449, 1338, 1028, 965, 895, 741.

EIMS m/z (%) 248 (M$^+$, 32), 215 (17), 175 (100), 130 (27).

FABMS m/z (%) 249 (M$^+$ + 1, 70), 175 (100), 130 (10).
HREIMS m/z calcd. for 248.0442 (C₁₂H₁₂N₂S₂), found 248.0445.

Methyl 2-oxotryptamine carbamate (140)

Pyridinium bromide perbromide (200 mg, 0.6 mmol) was added in small portions to a solution of methyl tryptamine carbamate (4, 109 mg, 0.5 mmol) in CH₂Cl₂ (10 mL) at 20 °C. The reaction mixture was stirred at 20 °C for 20 min, basified with DBU (200 µL) and refluxed for 10 min. The solvent was evaporated under reduced pressure and the mixture separated on a column [silica gel, CH₂Cl₂-MeOH (95 : 5)] to afford 50 mg (43%) of methyl 2-oxotryptamine carbamate (140).

¹H NMR (300 MHz, CDCl₃) δ 8.75 (br s, 1H), 7.27 (d, J = 8 Hz, 1H), 7.20 (dd, J = 8, 8 Hz, 1H), 7.02 (dd, J = 8, 8 Hz, 1H), 6.88 (d, J = 8 Hz, 1H), 5.27 (br s, 1H), 3.62 (s, 3H), 3.32-3.51 (m, 3H), 2.00-2.24 (m, 2H).

¹³C NMR (75.5 MHz, CDCl₃) δ 180.4 (s), 157.2 (s), 141.4 (s), 129.2 (s), 128.1 (d), 124.1 (d), 122.6 (d), 110.0 (d), 52.1 (q), 43.9 (d), 38.4 (t), 30.6 (t).
FTIR (cm$^{-1}$) 3305, 1707, 1620, 1533, 1471, 1262, 751.

EIMS $m/z$ (%) 234 (M$^+$, 49), 202 (86), 146 (100), 117 (12).

CIMS $m/z$ (%) 235 (M$^+$ + 1, 100), 203 (57), 146 (41).

HREIMS $m/z$ calcd. for 234.1004 (C$_{12}$H$_{14}$N$_2$O$_3$), found 234.1006.

4.4.5 Spectroscopic data

Cyclobrassinin homologue metabolite (142)

![Chemical structure](image)

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 10.79 (br s, 1H, D$_2$O exchangeable), 7.31 (dd, J = 8, 8 Hz, 1H), 7.13-7.21 (m, 2H), 7.06 (d, J = 8 Hz, 1H), 4.31-4.48 (m, 1H), 4.22-4.31 (m, 1H), 2.81-2.90 (m, 1H), 2.44-2.56 (m, 1H), 2.41 (s, 3H).

$^{13}$C NMR (75.5 MHz, CDCl$_3$) $\delta$ 206.6 (s), 173.0 (s), 143.1 (s), 135.0 (s), 129.5 (d), 124.9 (d), 124.1 (d), 110.6 (d), 77.1 (s), 60.9 (t), 41.3 (t), 14.4 (q).
FTIR (cm$^{-1}$) 3144, 2926, 2862, 1618, 1587, 1500, 1468, 1433, 1346, 1218, 1000, 753.

EIMS $m/z$ (%) 248 ($M^+$ - 16, 36), 215 (23), 175 (100), 149 (32), 130 (32).

CIMS $m/z$ (%) 249 ($M^+$ -15, 100), 217 (21), 203 (33), 171 (19).

HREIMS $m/z$ calcd. for 248.0442 ($C_{12}H_{12}N_2S_2$), found 248.0436.
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