CHEMICAL INVESTIGATION OF PHYTOALEXINS AND PHYTOANTICIPINS: ISOLATION, SYNTHESIS AND ANTIFUNGAL ACTIVITY

A thesis submitted to the

College of Graduate studies and Research

in partial fulfillment of the requirements

for the degree of

Master of Science

In the

Department of Chemistry

University of Saskatchewan

By

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Abstract

The focus of my research was on the secondary metabolites produced by crucifer plants under stress and their biological activity against fungi. Both cultivated and wild plants were investigated to isolate phytoalexins and phytoanticipins, and determine their metabolite profiles.

The first chapter of this thesis describes cruciferous plants and their most important pathogenic fungi. These plants are divided into three groups: oilseeds, vegetables and wild species. The metabolites isolated from these plants and their biosynthetic studies are reviewed. In addition economically important necrotrophic fungi such as *Leptosphaeria maculans*, *Alternaria brassicae*, *Sclerotinia sclerotiorum* and *Rhizoctonia solani* are also reviewed along with their phytotoxins.

The second chapter of this thesis describes the detection, isolation, structure determination, syntheses of stress metabolites and biological activity of these metabolites against *L. maculans*, *S. sclerotiorum* and *R. solani*. The investigation of cauliflower led to the isolation of seven phytoalexins: 1-methoxybrassitin (**55**), spirobrassinin (**71**), isalexin (**64**), brassicanal C (**60**), caulilexins A (**106**), B (**107**), and C (**105**). The phytoalexins caulilexins A (**106**), B (**107**) and C (**105**) were reported for the first time. Caulilexin A (**106**), having a disulfide bridge, showed the highest activity against *S. sclerotiorum* and *R. solani* among the known phytoalexins. Similarly four phytoalexins: 1-methoxybrassitin, brussalexins A (**121**), B (**117**) and C (**118**) along with four metabolites: ascorbigen (**51**), diindolylmethane (**50**), 1-methoxy-3,3'-diindolylmethane (**119**) and di-(1-methoxy-3-indolyl)methane (**120**) were isolated from

Brussels sprouts. The phytoalexins brussalexins A (**121**), B (**117**) and C (**118**) are new metabolites. Brussalexin A (**121**) is the only cruciferous phytoalexins having an allyl thiolcarbamate functional group. The metabolite 1-methoxy-3,3'-diindolylmethane (**119**) is reported for the first time.

The investigation of brown mustard for polar metabolites led to the isolation of indole-3-acetonitrile (**76**) and spirobrassinin (**71**) along with isorhamnetin-3,7-diglucoside (**134**). Investigation of wild species such as Asian mustard, sand rocket, wallrocket, hedge mustard and Abyssinian mustard for production of stress metabolites led to the isolation of indole-3-acetonitrile (**76**), arvelexin (**84**), 1,4-dimethoxyindole-3-acetonitrile (**137**), rapalexins A (**138**) and B (**142**), methyl-1-methoxyindole-3-carboxylate (**59**) and metabolites bis(4-isothiocyanotobutyl)-disulfide (**139**), 5-(3-isothiocyanato-propylsulfanyl)-pentylisothiocyanate (**136**) and 3-(methylsulfinyl)-propylisothiocyanate (**135**).

Two metabolites were also isolated from Brussels sprouts and brown mustard; however, these structures are not yet determined. The metabolites 1,4-dimethoxyindole-3-acetonitrile (**137**) and 5-(3-isothiocyanato-propylsulfanyl)-pentylisothiocyanate (**136**) are reported for the first time.

Acknowledgement

I would like to thank my supervisor, Professor Maria Soledade C. Pedras for her supervison and consistent support in the course of this work. Her indispensable guidance, constant encouragement and thoughtful suggestions immensely contributed to the successful completion of this work.

I would like thank to my advisory committee for their advice and suggessions during the program. I would also like to acknowledge both the present and past members of the Pedras group. I wish to thank the Department of Chemistry and the University of Saskatchewan for financial support.

Finally, I am grateful to my grandfather, parents and siblings for supporting me over the years.

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Ac	acetyl
Ac_2O	acetic anhydride
ATP	adenosine 5'-triphosphate
BJ-125	virulent isolate of *Leptosphaeria maculans*
br	broad
calcd	calculated
CI	chemical ionization
conc	concentrated
^{13}C-NMR	carbon-13 nuclear magnetic resonance
cv	cultivar
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
EI	electron impact
Et	ethyl
Et_2O	diethyl ether
Et_3N	triethylamine
EtOAc	ethyl acetate
EtOH	ethanol
FTIR	fourier transformed infrared
Glc	glucose
HMBC	heteronuclear multiple bond correlation
^{1}H-NMR	Hydrogen-1 nuclear magnetic resonance
HMQC	heteronuclear multiple quantum correlation
HPLC	high performance liquid chromatography
HR	high resolution
HST	host-selective toxin
LC	liquid chromatography
m/z	mass/charge ratio
Me	methyl
MeI	methyl iodide
MeOH	methanol
MHz	megahertz
Min	minute(s)
MS	mass spectrum
MC	Multiple column chromatography
PDA	potato dextrose agar
PDB	potato dextrose broth
ppm	parts per million

RP	reverse phase
R_T	retention time
THF	tetrahydofuran
TLC	thin layer chromatography
TMS	tetramethylsilane
UV	ultraviolet
V	volume
Wt	weight

Chapter 1

1. Introduction

1.1 General objectives

This thesis will describe phytoalexins and phytoanticipins from both cultivated and wild crucifer plants. The main objectives of this research project were to isolate phytoalexins and phytoanticipins, and to determine their chemical structures and biological activities. These investigations also attempt to compare phytoalexin production by cultivated and wild cruciferous plants. Cultivated plants can be susceptible to diseases caused by phytopathogenic fungi that cause significant crop losses. On the other hand, some wild species are resistant to these pathogens. Therefore, research that develops a better understanding of the factors potentially involved in the resistance of plants to fungal diseases may facilitate the control of fungal pathogens.

This research involved:

I) Isolation of active secondary metabolites from both crucifer vegetables and wild species.

II) Syntheses of new compounds.

III) Bioassays of new compounds to determine their antifungal activity.

1.2 Cruciferous plants

A large number of economically important plants cultivated worldwide belong to the Brassicaceae (Cruciferae) family. This family contains approximately 13-19 tribes and Brassiceae is one of these tribes (Gomez-Campo, 1980). Brassiceae contain 3500 species distributed in 350 genera; the most economically important crops are from the *Brassica* genus. These plants are important as they are sources of edible roots, stems, leaves, flowers and buds as well as oilseeds (edible and industrial) and condiment crops. *Brassica* includes vegetables such as cauliflower, broccoli, kale, the oilseeds canola and mustard, and wild species such as rocket, hedge rocket etc. Epidemiological studies have shown that a high intake of brassica plant products is associated with a lower risk of chronic diseases, such as atherosclerosis and cancer (Gundgaard *et al*., 2002).

1.2.1 Oilseeds, vegetables and wild species

Brassica oilseeds are the third most important world source of edible oil after soyabean and palm (Downey & Robbelen, 1989). Among the most important *Brassica* oilseeds are rapeseed (*Brassica napus* and *B. rapa*) and Indian / brown mustard (*B. juncea*) that contain 40-50% of seed weight in oil. The oilseed *B. juncea* (brown mustard) is an important source of oil in South Asia (Gomez-Campo, 1999). Brown mustard is resistant to drought, acidity, basicity, salinity, smog, weeds, insects and blackleg disease. Some oils from *Brassica* species contain a significant proportion of erucic acid (45%, cis-13-docosenoic acid) that downgrade the oil quality to non-edible. The meal obtained after crushing the seeds contains glucosinolates which made it unsuitable as a livestock feed. As a result, rapeseed oil and meal were regarded as low

quality products in most countries in the western world (Stefansson, 1990). With a high content of erucic acid (45%), rapeseed oil was used only for plastic and lubricant manufacturing. Plant breeders have been able to alter the chemical composition of rapeseed oil and provided an oil seed containing lower erucic acid and glucosinolates. This double-low seed type was named as "canola" (a contraction of "Canadian" and "oil"). In Canada there are two types of "canola" grown: a short season, yellow seeded, polish (*B. rapa*) and a longer season, blacked seeded (*B. napus*) variety (Fahey *et al.,* 2001). Hence, canola containing less than 1% erucic acid and < 30 micromoles of glucosinolates per gram of meal is now economically competitive with other edible oil seeds. Canola oil contains higher (55-65 %) oleic acid (18:1) and lower (20-23%) linolenic acid compared to sunflower oil and soyabean oil. The residual seed meal contains 38-46% of protein which made it a source of protein supplement in animal feeds (Rosa, 1999).

The chlorophyll content of canola seeds downgrades the oil quality. Genetic transformation of *B. napus* and *B. rapa* seeds reduced chlorophyll, odor and colour of oil (Morissette, *et al*., 1998). Canola oil is used mainly for cooking, non-stick spray, margarine, and salad oil. Canola is one of the important crops in the Canadian prairies (Alberta, Manitoba, and Saskatchewan). Canola quality brown mustard varieties are also available since the genes conditioning low glucosinolate content have been transferred to that species (Stefansson, 1990). Hence the cultivation of brown mustard is growing rapidly in Canada. The mustard seeded area in Saskatchewan increased to 242.8 thousand hectares in 2002 from 133.5 thousand hectares in 2001 (Field Crop Reporting Series, 2002, Statistics Canada Field Crop Reporting Series, 1992-2001). As

canola is one of the staples in the Canadian prairies, the reduction of yield caused by pathogens is a key issue in production. The main diseases of canola caused by pathogenic fungi are blackleg, stem rot, black spot, and root rot, as discussed in section 1.2.2.

In the *Brassica* genus, the next most important group of crops are vegetables. *Brassica* species are the third most important source of vegetables in the developed countries and in third place in consumption (Rosa, 1999). Brassica vegetables include different types of cabbages such as white, red, savoy, swamp and Chinese, rutabaga, Brussels sprouts, kale, kohlrabi, turnip, radish, cauliflower and broccoli. These vegetables possess antioxidant, as well as anticarcinogenic activity (Verhoeven *et al*., 1997). The epidemiological evidence strongly suggests that consumption of brassica vegetables is associated with reduced risk of cancers of the colon, rectum and thyroid and cancer at other sites (Gliszczynska-Swiglo *et al.*, 2006, Mithen *et al*., 2000, Mehta, *et al*., 1995). *Brassic*a vegetables are also potential sources of vitamins, carotenoids, and polyphenols.

A number of wild crucifers are reported to be resistant to cruciferous fungi. Wild crucifers such as *Diplotaxis muralis*, *D. tenuifolia*, *Sisymbrium loeselii*, *Arabidopsis thaliana* were reported as *Leptosphaeria maculans* resistant plants. The cotyledons, leaves, and stems of these plants were inoculated with *L. maculans* and their high level resistance was observed (Chen & Seguin-Swartz, 1999). Though all cultivated brassica species are susceptible to blackspot disease, some crucifer wild species such as *Sinapis alba* (white mustard), *Camelina sativa* (false flax), *Alliaria petiolata, Barbarea vulgaris*, *B. maurorum* and *C. bursa-pastoris* (Shepherd's purse)

show high levels of resistance to this disease (Pedras *et al.*, 2003, Westman & Dickson, 1998, Chrungu, 1999). The genomic sequence of the wild crucifer *A. thaliana* was published (The Arabidopsis Genome Initiative, 2000) and other crucifer genomes are under investigation.

1.2.2 Fungal pathogens

Fungal diseases are a major concern in crucifer production in many parts of the world. Depending on the parasitism, fungi are divided into two groups: biotrophic and necrotrophic. Biotrophic fungi are unable to survive in a dead host; therefore, they keep hosts alive and use the host's genetic and cellular processes to multiply. On the other hand, necrotrophic fungi, also known as parasitoids because they use the host's tissue for their own nutritional benefit until the host dies from loss of needed tissues or nutrients. Although necrotrophic fungi are considered primitive in comparison to the more sophisticated biotrophs, these fungi must also be highly specialized to avoid or suppress host resistance responses. *Leptosphaeria maculans, Rhizoctonia solani, Sclerotinia sclerotiorum*, *Alternaria brassicae* and *A. brassicicola* are among the most important necrotrophic fungi of crucifers whereas *Albugo candida* (Pers.) Kuntze, *Peronospora parasitica* and *Plasmodiophora brassicae* are the most important biotrophic fungi of crucifers (Goyal *et al*., 1995, Casimiro *et al.*, 2006). *A. candida* is responsible for white rust and staghead diseases whereas *P. parasitica* and *P. brassicae* are responsible for mildew and clubroot diseases to cruciferous plants respectively. (Gupta *et al*., 2006, Manzanares-Dauleux *et al.*, 2000).

Blackleg disease of cruciferous plants is caused by *L. maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm. Blackleg is a serious problem for brassica oilseed production in many parts of the world. Epidemic outbreaks of this disease caused yield losses up to 80% in France during the 1950s and in Australia during the 1970s (West *et al*., 2001). In Canada, losses of canola caused by *L. maculans* can reach up to 50%. It attacks leaves, cotyledons, pods, stems, and roots of crucifers. Blackleg spreads through canola stubble containing fruiting bodies pycinidia and pseudothecia. Pycinidia and pseudothecia release dispersed pycinidiospores and air born ascospores which infect the new healthy tissues. In its primary stage, the fungus grows in the intercellular space of leaves without showing any symptoms. After these, lesions appear on leaves and the fungus invades the stem, kills the stem cortex cell that leads to lodging and death of the plant. Blackleg infection symptoms are lesions in the leaves dotted with numerous pycnidia (Howlett *et al*., 2001). Taxonomic studies of *L. maculans* isolates have divided them in two groups. Isolates that cause stem canker on *B. napus* have been named aggressive, virulent or A-group isolates, whereas isolates causing only superficial damage have been named non-aggressive, PG1, avirulent or B-group isolates. Generally, virulent isolates grow slowly and irregularly on V-8 juice agar culture but avirulent isolates grow faster in this culture. In Czapek's broth media, virulent isolates produced no water soluble pigments but avirulent isolates produced yellow pigments (Koch *et al*., 1989). Moreover the virulent isolate cultures are characterized by sirodesmin production whereas sirodesmins were not detected in the cultures of avirulent isolates (Pedras & Seguin-Swartz, 1990). According to amplified fragment length

polymorphism (AFLP) analyses of different *L. maculans*, the B-group has been classified into three subgroups: NA1, NA2 and NA3 (Koch *et al.,* 1991). Recently, NA1 isolate of B-group has been classified as *L. biglobosa* (Rimmer, 2006). Based on the diseases reaction isolates, A-groups are subdivided into pathogenicity groups PG2, PG3 and PG4 (Koch *et al*., 1991). PG2 and PG3 are virulent on *B. napus* cultivars Westar and Glacier, respectively, whereas PG4 is highly virulent on both Westar and Glacier. Another group of *L. maculans* isolates called, Mayfair-2 and Laird-2, were found in Saskatchewan. These isolates are virulent on *B. juncea,* but avirulent on *B. napus* and *B. rapa.* Mayfair-2 and Laird-2 produced metabolites similar to those of avirulent Polish isolates and were, therefore, assigned to the Polish isolate group (Pedras & Biesenthal, 2000).

To invade the plant, fungi produce secondary metabolites which are toxic to plants, named phytotoxins. Based on the reactions of host plant, these toxins are classified into host-selective (HST) and non-selective phytotoxins (Graniti, 1991). Host-selective phytotoxins affect only the susceptible plants, but non-selective phytotoxins damage a broad range of plants. Phytotoxins interact with plants at the cellular level, causing dysfunction of plasma membrane and other organelles. They can also cause a rapid and dose dependent increase in electrolyte loss from cells (Khomoto & Otani, 1991). Phytotoxins were isolated from A-group of *L. maculans*. The first isolated non-host selective phytotoxin was sirodesmin PL (**1**) and later a host-selective phytotoxin phomalide (**2**) was isolated from 30-60 hour old cultures of blackleg fungus (Pedras *et al.*, 1993). Another phytotoxin, depsilairdin (**3**) isolated from Laird 2 isolate, causes strong necrotic lesions only on brown mustard leaves

(Pedras *et al*, 2004b). Similarly, polanrazine A (**4**) and phomalairdenone A (**6**) were reported from virulent isolates, whereas phomapyrone A (**5**) and phomapyrone F (**7**) were also isolated from both Mayfair 2 and Laird 2. Phomapyrone A (**5**) was isolated from avirulent isolates (Fig **1**, Pedras *et al*., 2005a).

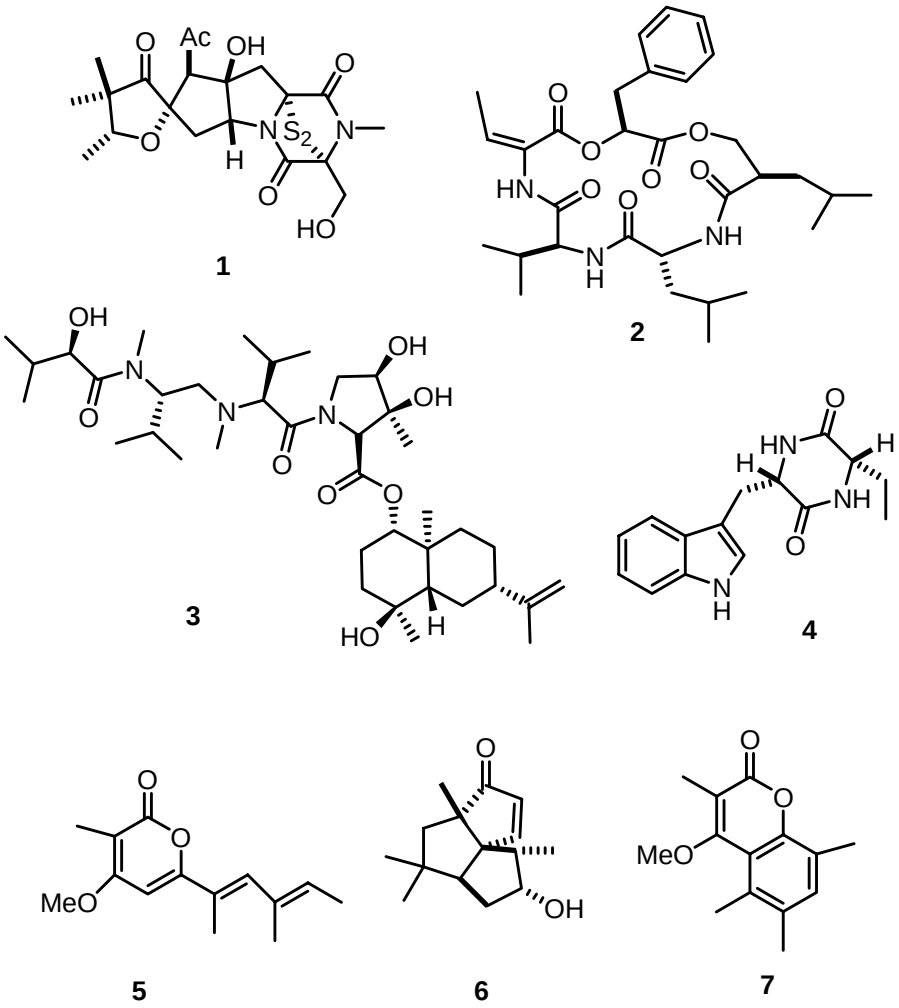
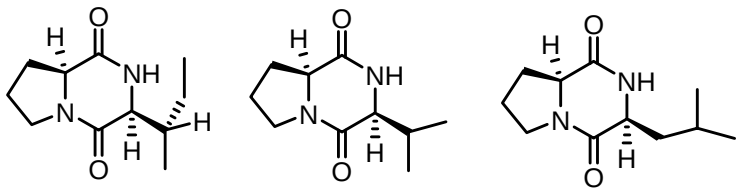


Fig **1.** Phytotoxin sirodesmin PL (**1**), phomalide (**2**), depsilairdin (**3**), polanrazine A (**4**), phomapyrone A (**5**), phomalairdenone A (**6**) and phomapyrone F (**7**) from *Leptosphaeria maculans* (Pedras *et al.*, 1993, Pedras *et al*, 2004b, 2005a,b).

Rhizoctonia solani Khun [teleomorph: *Thanatephorus cucumeris* (Frank) Donk] fungus causes different diseases, such as damping off, root rot, wirestem, bottom rot,

head rot, and root rot to cruciferous crops (Kataria & Verma, 1992). *R. solani* primarily attacks below ground plant parts, such as seeds, hypocotyls and roots, but is also capable of infecting above ground parts of plants such as stem, fruits and leaves. Damping off and root rot are widespread diseases in the Canadian prairies. Damping off occurs when germinating seedlings are infected prior to or just after emergence. On hypocotyls, lesions are reddish-brown, sunken and darken. The fungus can survive in soil and plant residues over the winter as mycelium or sclerotia. Root rot disease is observed all over the Canadian prairies. In Alberta, it is recognized as a serious disease (Kataria & Verma, 1992). According to hyphal anastomosis behavior, *R. solani* have been divided into 12 anastomosis group named AG1-11 and BI. Depending on the anastomosis frequency and thiamine requirement, AG2 was divided into three subgroups: AG2-1, AG2-2, and AG-2-3. Based on pathogenicity and cultural morphology, AG2-2 was divided into two intraspecific groups (ISGs), distinguished as IIIB and IV. The culture type IIIB isolates can grow at 35°C, but VI isolates cannot (Hyakumachi *et al.*, 1998). AG2-1 and AG4 are mainly responsible for canola and rape seed infection in Canadian prairies. The isolates AG2-1 are more virulent to canola and rapeseed plants than isolates AG4. The AG2-1 and AG4 isolates from seedlings were more virulent than the same isolates from adult plants (Kataria & Verma, 1992). The available methods to control the damping off and root rot disease are crop rotation and seed treatment with chemical fungicides. No phytotoxic compounds have been reported to date, but other metabolites (**8**), (**9**) and (**10**) have been reported from this fungus (Fig **2,** Pedras *et al.*, 2005c).

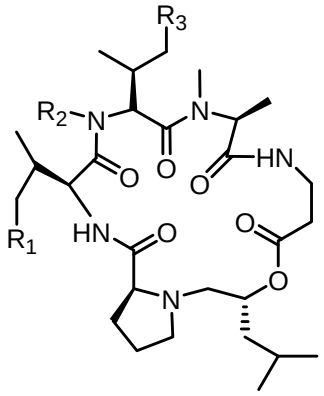


8 **9** **10**

Fig **2**. Metabolites isolated from the root rot fungus *Rhizoctonia solani* (Pedras *et al.*, 2005c).

Alternaria black spot and dark leaf spot diseases are among the most destructive diseases of brassica crops worldwide and are caused by *Alternaria* spp. Four species of *Alternaria* pathogen named *Alternaria brassicae*, *A. brassicicola, A. alternata* (Fr.) Keissler and *A. japonica* Yoshii are pathogenic on *Brassica* spp. *A. brassicae* and *A. brassicicola* are the most important economically and both are found in crucifer growing areas. Alternaria black spot, caused by *A. brassicae* usually occur during warm and moist weather. The hosts of these species include oleiferous, vegetables, wild crucifers and some other non-cruciferous plants. Though both species may occur on the same crop, *A. brassicae* normally associates with the oleiferous *B. juncea, B. napus* and *B. rapa* and *A. brassicicola* associates with the vegetable *B. oleracea* L. (Humpherson-jones & Phelps, 1989). Infected seeds and infected weeds are the direct source of infection of the host plant. The major symptom of *Alternaria* species is the leaf spotting. Yellow-brown spots with target-like concentric rings appear on leaves, as well as dark brown sunken spots on heads of Brussels sprouts, broccoli and cauliflower (Gomez-Campo, 1999). Although commercial *Brassicas* are susceptible to *A. brassicae,* they are different in the degree of susceptibility (Jasalavich, *et a*l., 1993). *B. juncea* and *B. rapa* are more susceptible to *A. brassicae* than *B. napus* and *B. carinata*.

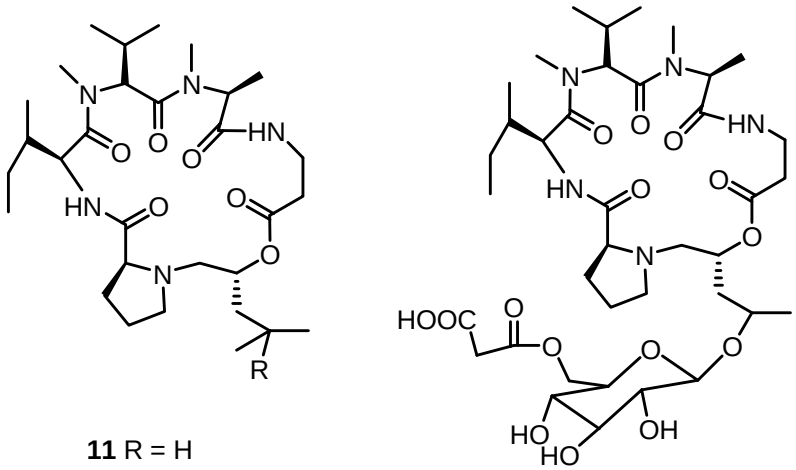
The fungus persists on crop debris and wild crucifers and/ or in seeds. In unfavorable conditions, e.g. winter, *Alternaria* species survive on the infected crop residue, wild species, and seeds. Hot-water treatment of seed reduces both internal infection and external infestation of seed, while protective fungicide treatment will only control spores on the seed surface. Crop rotation with non cruciferous and eradication of cruciferous wild species also help to control this infection (Howard *et al*., 1994). Four phytotoxins destruxin B (**11**), homodestruxin B (**12**), desmethyldestruxin B (**13**) and destruxin B_2 are known from *A. brassicae.* The major phytotoxic compound produced by *A. brassicae* is destruxin B (**14**). The cyclodepsipeptide destruxin B causes chlorotic and necrotic foliar lesions in many species of cruciferous plants (Fig **3,** Pedras *et al.* 2003a).



11 R_1 =CH_3, R_2=CH_3, R_3=H
12 R =CH_3, R_2=CH_3, R_3=CH_3
13 R_1 =H, R_2=CH_3, R_3=CH_3
14 R_1=H, R_2=CH_3, R_3=H

Fig **3**. Phytotoxins destruxin B (**11**), homodestruxin B (**12**), desmethyldestruxin B (**13**), and destruxin B_2 (**14**) from *Alternaria brassicae* (Pedras *et al*., 2003a).

Both susceptible and resistant plants transformed destruxin B (**11**) to hydroxydestruxin B (**15**). Then the metabolite hydroxydestruxin B (**15**) was further transformed by glucosylation and malonylation to hydroxydestruxin B-β-D-glucopyranoside (**16**) and (6'-*O*-malonyl)hydroxydestruxin B-β-D-glucopyranoside (**17**), respectively. Both hydroxylation and glucosylation reactions occur in susceptible and resistant plants. In resistant species, glucosylation was the rate limiting step, whereas in susceptible species, hydroxylation was the rate limiting step (Fig **4,** Pedras *et al*., 2003a). As *A. thaliana* was the first flowering plant having the genomic sequence known, research possibilities to increase resistance to diseases has made the combination of this plant with *A. brassicicola* fungus an important model system for the investigation of polygenic resistance (Tierens, *et al*., 2002, Cramer, *et al*., 2006, Thomma, *et al.*, 1999).



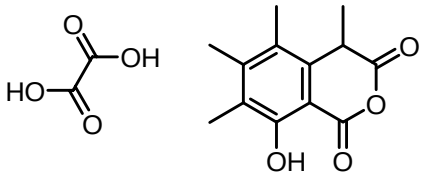
11 R = H
15 R = OH
16 R = glc

17

Fig 4. Metabolism of the host selective toxin destruxin B (**11**) by crucifers: hydroxydestruxin B (**15**), hydroxydestruxin B-β-D-glucopyranoside (**16**), and 6'-O-malonyl)destruxin B-β-D-glucopyranoside (**17**) (Pedras *et al*., 2003a).

Sclerotinia sclerotiorum (Lib.) de Bary causes different soil-borne diseases such as sunflower wilt, root rot of pea and timber rot of tomato, and airborne diseases such as stem rot of canola, cottony soft rot of carrot, lettuce drop of lettuce and white mould on beans (Purdy, 1979, Bardin & Huang, 2001). More than 408 species in 278 genera are the host of this fungus (Boland & Hall, 1994). All types of canola, brown mustard, rapeseed and white mustard are susceptible to *Sclerotinia* stem rot. Because of *Sclerotinia* stem rot, the yield losses of canola and rapeseed can reach up to 50%. The initial symptom of *S. sclerotiorum* is the presence of cottony white mycelial growth on the surface of plants such as cabbage, lettuce, carrot, and canola. Within this fluffy white mass, dense white bodies of fungus form. These bodies become black and are called sclerotia. The pigment of sclerotia is due to the presence of melanin, which plays an important role in protecting fungi from adverse biological conditions and harsh environmental situations (Starratt *et al*., 2002). The size of sclerotia range from 2 to 10 mm in length and can survive for 4 to 5 years in the soil (Kohn *et al*., 1995, Bardin & Huang, 2001). After the dormant period when the moisture and temperature are suitable, the sclerotia germinate, and subsequently, the apothecia form to liberate ascospore into the air. The apothecia produce enormous numbers of ascospores that are blown out and cause primary infections (Kohn *et al*., 1995). The air disseminated ascospores adhere to the surface of canola petals. Ascospores then invade the green leaves of stem and penetrate the stem to form lesions in which sclerotia eventually develop. Once the fungus is established, it continues vegetative growth as long as there is sufficient moisture. Severely infected crops lodge and the seed pod shattered result in a total crop loss. Though stem rot is a crucial problem in Saskatchewan and western

Canada, no cultivar of canola resistant to this disease is commercially available. *S. sclerotiorum* attacks have negative impact on the quality and production of crops. Due to the lack of adequate genetic resistance in oilseed, disease control therefore has to rely largely on crop rotation, use of certified seeds, removal of infected stubble, and application of fungicides. A few metabolites were isolated from this fungus, but only two phytotoxins are reported to date. Oxalic acid (**18**) was reported to be a pathogenicity determinant for *S. sclerotiorum*; mutants unable to produce oxalic acid were not pathogenic. But in *B. napus*, *B. juncea* and *S. alba,* oxalic acid (**18**) did not show any phytotoxicity, whereas sclerin (**19**) also produced by this fungus, showed toxicity (Fig **5,** Pedras & Ahiahonu, 2004).



18 **19**

Fig **5.** Metabolites oxalic acid (**18**) and sclerin (**19**) from *Sclerotinia sclerotiorum* (Pedras & Ahiahonu, 2004).

1.2.3 Secondary metabolites

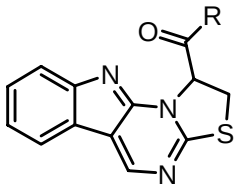
The complete set of chemical reactions that occur in living organisms to make them grow, reproduce, maintain their structures and respond to their environment are called metabolism (Mann, 1987). Metabolism is usually divided into two categories: primary metabolism and secondary metabolism. Primary metabolism encompasses

reactions involving those compounds which are formed as a part of the normal anabolic and catabolic processes and take place in most cells of the organism. On the other hand, processes that result in many of the chemical changes of compounds and which are required for secondary utilities are known as secondary metabolism. Secondary metabolites are synthesized from primary precursors. These, secondary metabolites include the following: a) polyketides derived from acetate-malonate; b) terpenoids derived from acetate-mevalonate; c) aromatic amino acids and polyphenyles derived from shikimic acid; d) alkaloids derived from amino acids; e) metabolites derived from a/the mixed biogenetic origin (Mann, 1987).

1.2.3.1 Chemical defenses

Pathogen attacks reduce the quality and quantity of crops all over the world. When plants come under fungal attack, they respond by blocking or delaying the advancement of the invader. These mechanisms include preformed physical and chemical barriers, as well as inducible defenses, such as strengthening the cell wall, the hypersensitive response, development of systemic acquired resistance (SAR) and synthesis of antimicrobial molecules (Grayer & Harbone., 1994). The physical barriers that plants produce are due to the lignification process in the cell wall to prevent pathogens from penetrating into the plant tissues. Epicuticular wax may also contribute to resistance. However, some microorganisms are able to overcome the defense system and infect plant tissues. To fight against pathogenic fungi, plants also produce a broad range of secondary metabolites such as phytoanticipins (VanEtten *et al*., 1994) and phytoalexins (Pedras *et al.*, 2003). Two indole based compounds, 1,2-dihydro-3-thia-

4,10,10b-triaza-cyclopenta[.a.]fluorine-1-carboxylic acid (**20**) and [(1,2-Dihydro-3-thia-4,10,10b-triaza-cyclopenta[a]fluorene-1-carbonyl)-amino]-acetic acid (**21**) were isolated from the surface of *B. oleracea* cv. *botrytis* leaves; these compounds stimulated very effectively oviposition in the cabbage root fly, *Delia radicum* (Fig **6**, Hurter *et al.*, 1999).



20 R = OH
21 R = NHCH2COOH

Fig **6**. Metabolites 1,2-dihydro-3-thia-4,10,10b-triaza-cyclopenta[.a.]fluorine-1-carboxylic acid (**20**) and [(1,2-Dihydro-3-thia-4,10,10b-triaza-cyclopenta[a]fluorene-1-carbonyl)-amino]-acetic acid (**21**) are isolated from cauliflower infested by *Delia radicum* (Hurter *et al.*, 1999)

Phytoanticipins

The term 'phytoanticipin' was proposed by Mansfield and defined as "low molecular weight antimicrobial compounds that are present in the plant before challenge by microorganisms or are produced after infection solely from preexisting constituents" (VanEtten, *et al.,* 1994). A large number of constitutive plant compounds have been reported to have antifungal activity. These compounds include major classes of secondary metabolites such as terpenoids, long chain fatty acids, saponins, phenols and phenolic glycosides, unsaturated lactones, sulfur compounds, cyanogenic

glycosides, and glucosinolates (Osbourn, 1996a). These secondary metabolites are present in a wide range of plant species. A wild species, *Crambe abyssinica* (Abyssinian mustard), is getting more attention due to production of triglycerides (refined oil), erucic acid, behenic acid, erucyl acid wax esters and fatty acids. Two constitutive compounds crambene (**28**) and phenylethyl cyanide isolated from seed meal of Abyssinian mustard are used as insecticides against house fly (Bondioli, *et al.*, 1997, Peterson *et al*., 2000, Niedoborski, *et al*., 2001). It was shown that two compounds from Brussels sprouts crambene (**28,** Fig **8**) and indole-3-methanol elevate quinine reductase, glutathione transferase (phase 2 detoxification enzymes) and CYP1A activity individually and synergistically (Staack *et al.,* 1998). More than 120 glucosinolates were found in 16 plant families (Hailkier, 1999). Glucosinolates are present in all investigated brassica species and form the main class of phytoanticipins in the crucifer family. *Brassica* vegetables can contain glucosinolates up to 1% of dry weight (Rosa *et al*., 1997). The structure of glucosinolates contains a β-D-thioglucoside-N-hydroxysulfate group and a side chain (Lee *et. al*., 2006). Most of the side chains are either straight or branched carbon chains having double bonds, hydroxyl, carbonyl or various sulfur groups. One third of all glucosinolates side chains contain a sulfur atom in various oxidation states such as methylthioalkyl-, methylsulfinylalkyl or methylsulfonylalkyl. Twenty three different glucosinolates were reported from *A. thaliana* (Haughn *et al.*, 1991). Glucosinolates are divided into three groups depending on the side chain nature e.g. aliphatic, aromatic and heteroaromatic. Glucosinolates are derived mainly from seven amino acids (alanine, valine, leucine, isoleucine, phenylalanine, tyrosine and trytophan). Glucosinolates have been used as

taxonomic markers to support evolution based classification of plants. For example, methyl glucosinolate is absent in the Brassicaceae, but is a distinctive component in the Capparaceae family. Some glucosinolates such as glucobrassicin (**22**), hydroxylGluco-brassicin (**23**), 4-methoxty glucobrassicin (**24**), neoglucobrassicin (**25**), aliphatic glucosinolate and others (Fig **7**) were reported from cauliflower (Tian *et al.,* 2005). Aliphatic and aromatic glucosinolates, and nitrile compounds were reported from Brussels sprouts (*B. oleracea* var. *gemmifera*). These are sinigrin (**29**), glucobrassicin (**22**), allylnitrile (**30**) and sulforaphane (**31**) (Liang *et al*., 2005, Smith *et al.*, 2005). Glucobrassicin (**22**), neoglucobrassicin (**23**) and sulforophane (**31**) were reported at high levels in broccoli (Fig **7**, Fig **8**, Fahey, *et al*., 2001).

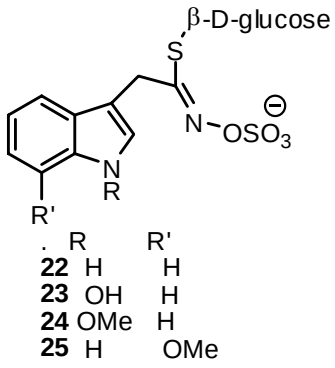
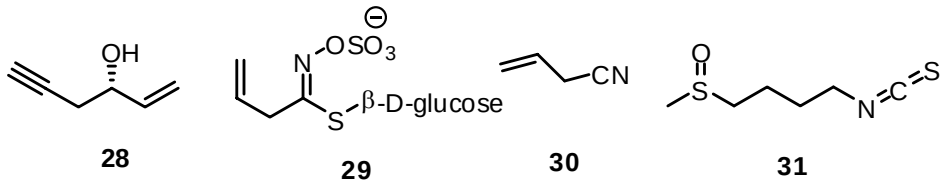


Fig **7**. Phytoanyicipins glucobrassicin (**22**), hydroxyglucobrassicin (**23**), 4-methoxty glucobrassicin (**24**) and neoglucobrassicin (**25**) from cauliflower (Tian *et al.,* 2005).



28 **29** **30** **31**

Fig **8**. Phytoanticipins crambene (**28**), sinigrin (**29**), allylnitrile (**30**), sulforaphane (**31**) from Brussels sprouts (Liang *et al*., 2005, Smith *et al.*, 2005).

In 1962, Underhill and co-workers first reported that glucosinolates are biosynthesized from amino acids. Feeding experiments with radioactive ^{14}C labelled amino acids and ^{14}C-acetate to horseradish, nasturtium and watercress resulted in the incorporation of ^{14}C into glucosinolates (Underhill *et al*., 1962, Chisholm & Wetter., 1964). Glucosinolate biosynthesis involves three major steps: 1) side chain elongation, 2) glycone biosynthesis and 3) side chain modification. The first stage of biosynthesis of glucosinolates involves the conversion of amino acids to aldoximes (**32**) (Mikkelsen, *et al.*, 2002, Mithen *et al*., 2000). It is believed that aldoximes (**33**) are converted to *S*-alkyl-thiohydroxamic acid (**35**) by insertion of sulfur from cysteine through nitro intermediates **34** (Mikkelsen, *et al.*, 2002). *S*-glycosyl transfer from UDP-glucose and sulfonation by the high energy sulfate donor, 3'-phosphoadenosine-5-phosphosulfate (PAPS) is known to occur. *S*-Glycosylation of thiohydroximic (**36**) is catalysed by UDP:thiohydroximate glucosyltransferase to yield a desulfoglucosinolate (**37**) (Guo & Poulton, 1994). The final step is the sulfation of

desulfoglucosinolate (**37**) through a soluble 3'-phosphoadenosine-5-phosphosulfate (PAPS): desulfoglucosinolate sulfotransferase to yield a glucosinolate (**38,** Fig **9**).

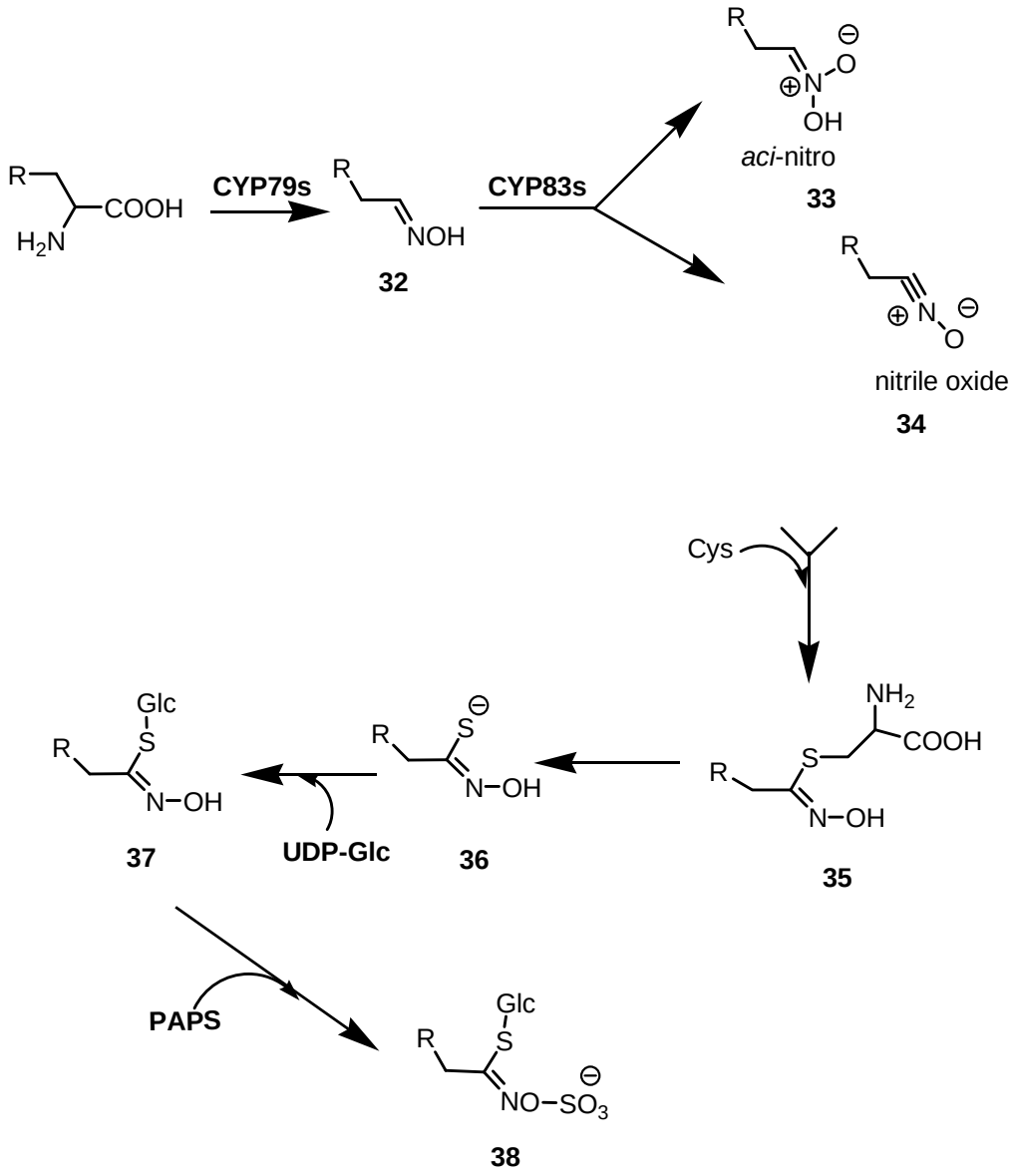


Fig **9**. Glucosinolate biosynthetic pathway (Mikkelsen, *et al*., 2002, Fahey, *et al*., 2001).

The glucosinolate degradation product allyl isothiocyanate is largely responsible for the flavor of mustard and horseradish as well as bitter taste for condiment crops, whereas the glucosinolates sinigrin and progoitrin confer bitterness on Brussels sprouts and other *Brassica* vegetables (Mithen *et al*., 2000). The glucosinolates are converted to isothiocyanates upon wounding of the plant, by mastification of fresh vegetables, or by tissue damage (Rosa et al., 1997). The tissue damage releases a glycoprotein myrosinase (EC 3.2.3.1) which coexist with glucosinolates, but are physically separated from each other. It was thought that myrosinase is localized in specialized 'myrosin' cell (Drozdowska *et al.*, 1992). It has been suggested that myrosin cells are formed at an early state of leaf development and then no new cells are produced (Pocock *et al*., 1987). Although this enzyme is present in dormant mature seeds, it is normally sequestered within aqueous vacuoles of cells (Bones & Rossiter, 1996, 2006).

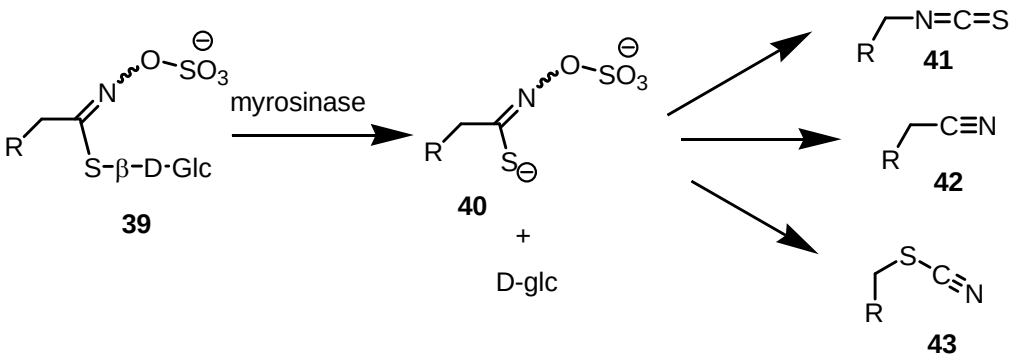


Fig **10**. Scheme of glucosinolate hydrolysis (Mithen *et al*., 2000).

When tissue disruption occurs, glucosinolates from vacuoles are released along with myrosinase, which results in the release of glucose to leave unstable intermediates, thiohydroxamate-o-sulfonates (**40**). This intermediate further degrades to produce isothiocyanates (**41**), nitriles (**42**) and thiocyanates (**43,** Fig **10**). Lossen-type rearrangement of thiohydroxamate-O-sulfonates (**40**) produces isothiocyanates (Mithen *et al*., 2000). The enzyme catalyzed hydrolysis of glucobrassicin (**44**) yields unstable thiohydroxamate-O-sulfonates (**45**). At pH 7, this intermediate further degrades to the corresponding alcohol and may condense to form diindolylmethane; however, in more acidic pH 3, it gives indolyl-3-carbinol (**49**), indolyl-3-acetonitrile (**46**) and elemental sulfur (Fig **11,** Agerbirk *et al.*, 1998). In reaction with L-ascorbate, the intermediate **48** produced ascorbigen (**51**). Though antifungal metabolites indolyl-3-acetonitrile (**46**) and elemental sulfur are released from glucobrassicin (**44**) in response to stress, these are phytoanticipins because the substrate and myrosinase enzyme are present in the healthy cell.

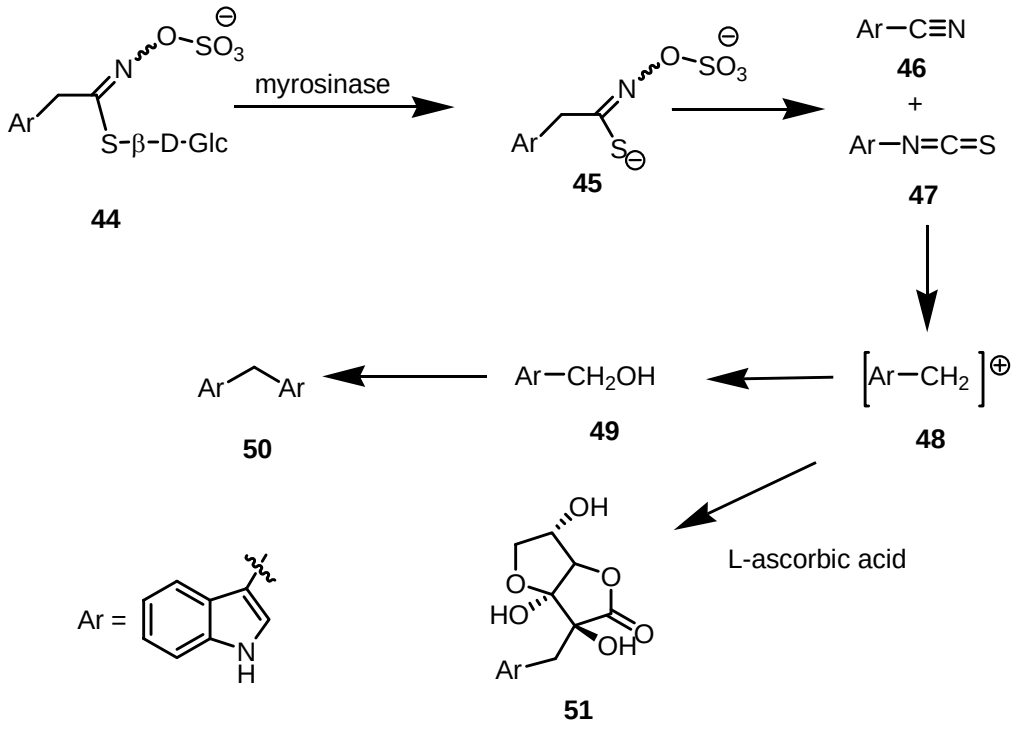


Fig **11**. Myrosinase-catalysed hydrolysis of glucobrassicin (Agerbirk *et al.*, 1998).

Although glucosinolates are known as flavor precursors, the main reason of glucosinolate research stemmed from the adverse effect that these compounds had on animals. However, studies showed that the inclusion of high glucosinolate meal reduced feed intake, enlarged thyroid, caused abnormalities of liver and kidneys, and reduced growth and reproductive performance (Mawson *et al*., 1993, Mawson *et al*., 1994, Mawson *et al*., 1994a, Bell, 1993). Studies on the metabolic detoxification of carcinogens showed three stages of detoxification: activation of carcinogen by oxidation (Phase I); conjugation to a more polar strcuture (Phase II); and the eventual excretion and transport out of the cell (Phase III). *Brassica* diets have been shown to increase the oxidative metabolism and to facilitate glucuronide conjugation (Phase II) (Johnson *et al*., 1995, Talalay *et al*., 1995). It was shown that the glucotropaeolin

breakdown product, benzyl isothiocyanate inhibited the induction of mammary tumours by 7,12-dimethylbenzlanthracene (DMBA) when administered orally to rats two hours before treatment with the carcinogen (Wattenberg 1977). The major glucosinolate breakdown products in the leaves of *Brassica* plants are isothiocyanates. Two isothiocayantes allyl-(2-propenyl)isothiocyanate and 2-butenylisothiocyanate were found to be highly toxic to fungi. The fact that glucosinolate breakdown products are also effective against some pathogens, which are non pathogenic to *Brassica*, led to their use as fungicides for control of cereal diseases and post harvest pathogens of fruits and vegetables (Osbourn, 1996).

Phytoalexins

The term 'phytoalexin' was first introduced by Muller and Borger (1940) from the Greek words φυτον = plant and αλεξειν = to defend. Probably phytoalexin production is one of the most studied defense responses of plants to microbial infection. Over a period of time, the definition of the term has been modified according to new evidence. The widely accepted definition is that phytoalexins are low molecular weight secondary metabolites locally produced *de novo* in plants at the time of both biotic and abiotic stress (Pedras *et al.*, 2003). This is why antifungal peptides and proteins produced by plants do not belong to this group. At the beginning, phytoalexin research was devoted to find novel natural fungicides, but the antifungal activities of most phytoalexins were much lower than that of commercial fungicides. Phytoalexins from Crucifers have been studied for over two decades (Pedras *et al.*, 2006). These works covered mainly vegetables, wild plant species and most of the brassica oilseed plants.

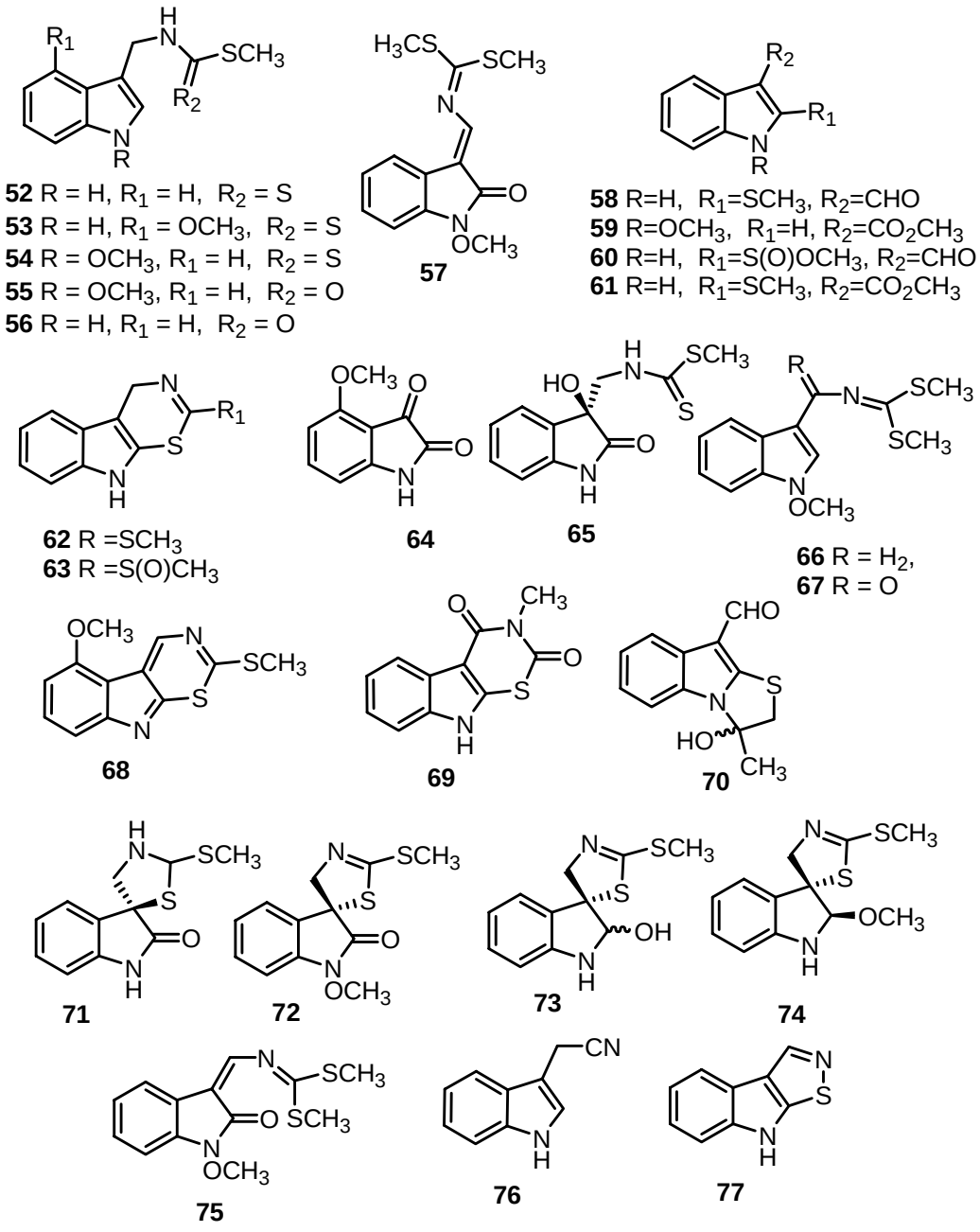


Fig **12**. Phytoalexins from cultivated crucifers: brassinin (**52**), 4-methoxybrassinin (**53)**, 1-methoxybrassinin (**54**), 1-methoxybrassitin (**55**), brassitin (**56**), wasalexin A (**57**), brassicanal A (**58**), methyl 1-methoxyindole-3-carboxylate (**59**), brassicanal C (**60**), brassicanate (**61**), cyclobrassinin (**62**), cyclobrassinin sulfoxide (**63**), isalexin (**64**), dioxibrassinin (**65**), 1-methoxybrassenin A (**66**), 1-methoxybrassenin B (**67**), dehydro-4-methoxyclobrassinin (**68**), rutalexin (**69**), brassicanal B (**70**), spirobrassinin (**71**), 1-methoxyspirobrassinin (**72**), 1-methoxyspirobrassinol (**73**), 1-methoxyspirobrassinol methyl ether (**74**), wasalexin B (**75**), indolyl-3-acetonitrile (**76**) and brassilexin (**77**).

A significant characteristic of phytoalexins reported form crucifers is that they are indole derived compounds (Pedras *et al.*, 2003, 2007, Fig **12**).

The first phytoalexins isolated from cruciferous plants were brassinin (**52**), cyclobrassinin (**62**) and 1-methoxybrassinin (**54**). These three phytoalexins were isolated from Chinese cabbage *Brassica campestris* L. ssp *pekinesis* elicited by *P. cichori* (Takasugi *et. al.* 1986). Since then, more than thirty phytoalexins were reported from 25 species (Pedras *et al.*, 2003, Pedras *et al.*, 2007). Out of all, 23 phytoalexins (**52-77**) were isolated from 12 cultivated plants (Fig **12**) and 8 phytoalexins were isolated only from 13 wild species. Some phytoalexins such as spirobrassinin (**71),** 1-methoxyspirobrassinin (**72),** indolyl-3-acetonitrile (**76)**, brassilexin (**77)**, cyclobrassinin **(62)**, cyclobrassinin sulfoxide (**63)**, 1-methoxybrassinin (**55)**, and wasalexin A (**57)** were reported from both cultivated and wild species. Five phytoalexins brassilexin (**77**), cyclobrassinin (**63**), cyclobrassinin sulfoxide (**64**), indole-3-acetonitrile (**76**) and spirobrassinin (**71**) were isolated from brown mustard elicited with *A. bassicae* and *L. maculans* (Pedras *et al*., 2002a). From *Thlaspi arvense* another phytoalexin was isolated known as wasalexin A (**57**) which was reported before from *Wasabi japonica* syn. *Eutrema wasabi* (Pedras *et al.,* 1999a, 2004c). The two species are from different genus, but both of them are resistant to *L. maculans.* Rutalexin (**69**) was isolated from kohlrabi, but the wrong structure was assigned (Gross *et al.*, 1994, Pedras *et al*., 2004g). On the other hand, erucalexin (**85**), isolated from *Erucastrum gallicum* (dog mustard), is a unique phytoalexin having a carbon substitutent at C-2 position instead of the common C-3 substitution observed in all other crucifer phytoalexins (Fig **13,** Pedras *et al.,* 2006, 2000c).

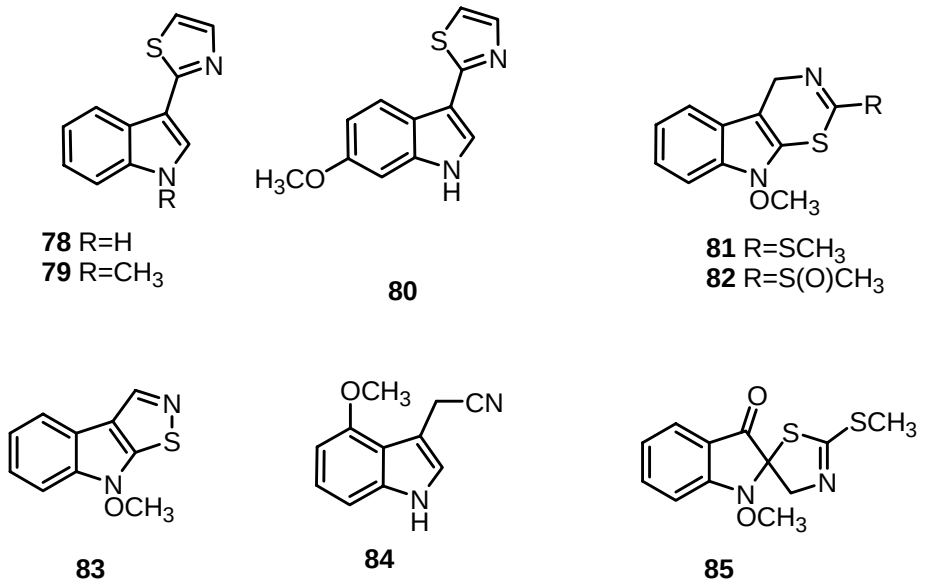


Fig **13.** Phytoalexins from wild crucifers: camalexin (**78**), 1-methylcamalexin (**79**), 6-methoxycamalexin (**80**), sinalbin B (**81**), sinalbin A (**82**), sinalexin (**83**), arvelexin (**84**), erucalexin (**85**).

Brassinin (**52**) is an important phytoalexin, not only for its antifungal activity, but also because it is a precursor of many phytoalexins (Pedras *et al*., 2007). Like some pesticides and herbicides, brassinin has a dithiocarbamate group. Brassinin (**52**) was predicted to derive from glucobrassicin (**22**). Studies on UV-irradiated turnip tissue that had been fed deutereted compound showed that both brassinin (**52**) and glucobrassicin (**22**) are biosynthesized from *S*-tryptophan; however, glucobrassicin was not a precursor of brassinin (**52**) (Pedras *et al*., 2007). The methyl group of brassinin (**52**), cyclobrassinin (**62**), and spirobrassinin (**71**) were originated from (*S*)-methionine. Incorporation studies with ^{35}S-cysteine showed that the thiocarbonyl sulfur atom of brassinin originated from (*S*)-cysteine. Administered [methyl $^{2}H_3$]-brassinin to UV-irradiated turnip roots indicated effective incorporation of deuterium

into spirobrassinin (**71**) and cyclobrassinin (**62**) (Monde *et al.,* 1994). Feeding experiments were conducted to determine whether cyclobrassinin (**62**) was a precursor of spirobrassinin. However, [methyl $^{2}H_3$]- cyclobrassinin was not incorporated into spirobrassinin. Experiments done by Pedras and co-workers using *Brassica carinata*, elicited with *L. maculans* (blackleg fungus), showed that brassilexin (**77**) is formed from brassinin (**52**) through cyclobrassinin (**62**) (Pedras *et al.,* 2002a, 2003). These results demonstrated that brassinin (**52**) is an advanced precursor of both cyclobrassinin (**62**) and spirobrassinin, and that spirobrassinin is biosynthesized directly from brassinin (**52**). Furthermore brassilexin (**77**) is biosynthesized from brassinin (**52**) via cyclobrassinin (**62**). Further studies have established that (*S*)-tryptophan (**86**) is converted to brassinin (**52**) via indole-3-acetaldoxime (**87**). It also demonstrated that indole-3-acetaldoxime (**87**) is the precursor of both brassinin (**52**) and glucobrassicin (Pedras *et al.* 2002a). Rutalexin (**69**), erucalexin (**85**) and 1-methoxyspirobrassinin (**72**) originated from cyclobrassinin (**62**) (Fig **14**, Pedras *et al*., 2004f, 2004g, Pedras & Okinyo, 2006a).

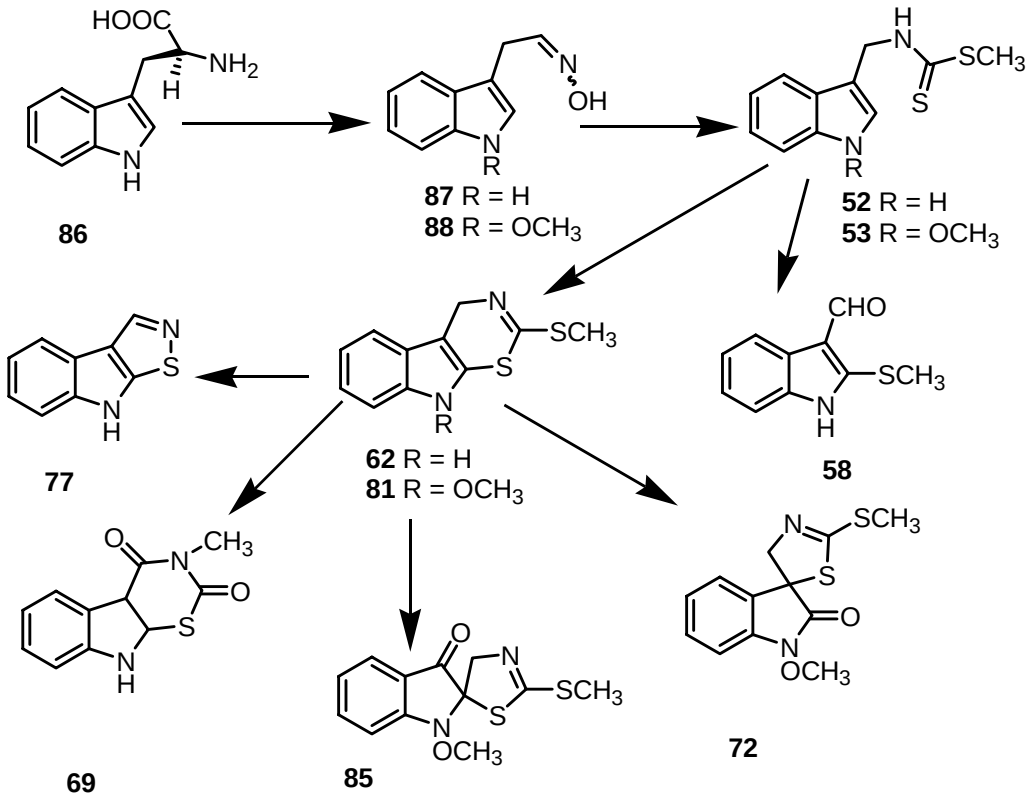


Fig **14**. Biosynthesis of major cruciferous phytoalexins (Pedras *et al*., 2007).

Recently, crucifer phytoalexins are getting more attention due to their chemopreventive activity (Mezencev *et al.*, 2003). It was found that brassinin, cyclobrassinin and spirobrassinin inhibited the formation of 7,12-dimethylbenz (a)anthracene-induced mammary lesions in a dose-dependent manner (Mertha *et al.*, 1994). In other experiments, it was found that 1-methoxybrassinin exerted the potent cytotoxic and apoptosis-inducing activity in Jurkat cells (Pilatov *et al.,* 2005). Camalexin (**78**) is one of the most discussed phytoalexins because the genome sequence of camalexin producing plant *A. thaliana* is published (The Arabidopsis Genome Initiative, 2000). It was shown that if the approximate concentration of camalexin reached 50-100 μM in necrotic area of wild type *A. thaliana*, the spore

germination and germ-tube elongation of most of the *Alternaria* reduced significantly (Sellam *et al*., 2007).

Detoxification of cruciferous Phytoalexins

Phytoalexins can inhibit the plant pathogen but pathogens can circumvent these plant chemical defenses through metabolic detoxification (Pedras & Ahiahonu, 2005). As a result plants become susceptible to the fungus. Crucifer phytoalexin detoxifications were studied using three cruciferous phytopathogenic fungus such as *L. maculans*, *S. sclerotiorum*, and *R. solani* (Pedras & Ahiahonu, 2005).

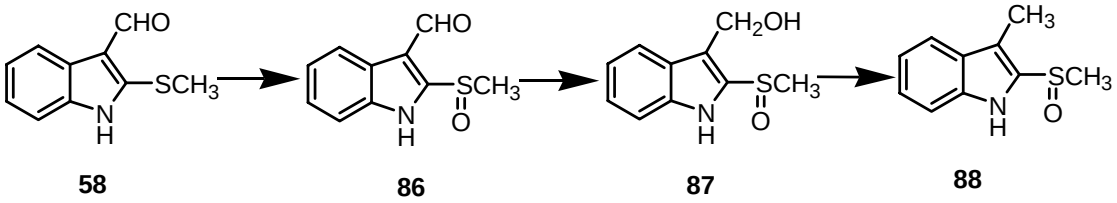


Fig **15**. Detoxification of brassicanal A (**58**) by *Leptosphaeria maculans* (Pedras & Ahiahonu, 2005).

Brassicanal A (**58**) was biotransformed by *L. maculans* to three metabolites: brassicanal A sulfoxide (**86**), 3-hydroxymethylindolyl-2-methylsulfoxide (**87**), and 3-methylindolyl-2-methyl-sulfoxide (**88**). The first step in the transformation of brassicanal A (**58**) involved oxidation of SCH_3 group yielding brassicanal A sulfoxide (**86**), which was subsequently reduced at aldehyde group to 3-hydroxymethylindolyl-2-methylsulfoxide (**87**) and then further to the 3-methylindolyl-2-methyl-sulfoxide (**88,** Fig **15**). The biotransformation of brassicanal A (**58)** was shown to be a

detoxification because the transformed metabolites (**86-88**) were significantly less antifungal to *L. maculans* than brassicanal A (**58**) (Pedras & Ahiahonu, 2005).

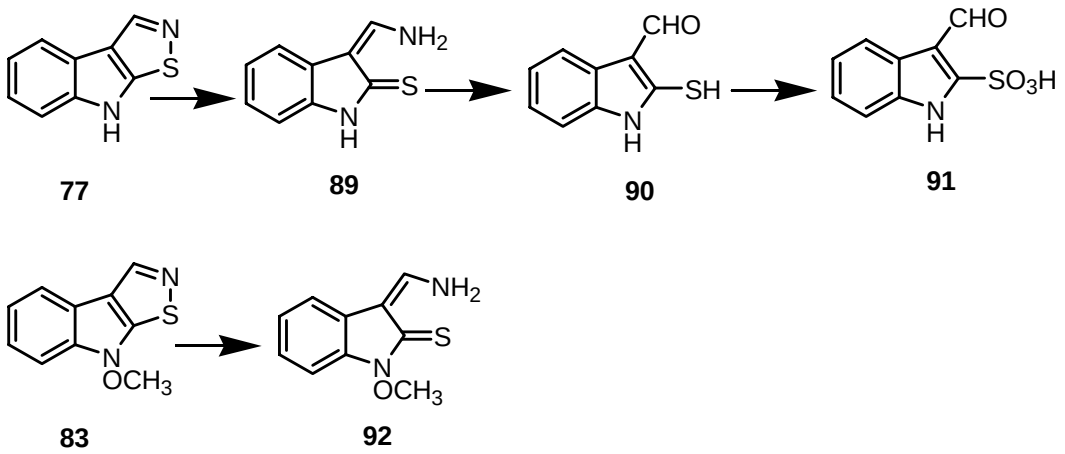


Fig **16**. Detoxification of brassilexin (**77**) and sinalexin (**83**) by *Leptosphaeria maculans* (Pedras & Suchy, 2005).

The transformation of brassilexin (**77**) and sinalexin (**83**) by *L. maculans*, involved reduction of isothiazole ring yielding 3-aminomethyleneindole-2-thione (**89**) and 1-methoxy-3-aminomethyleneindole-2-thione (**92**) respectively. Subsequently 3-aminomethyleneindole-2-thione (**89**) was hydrolyzed to 2-sulfanylindolyl-3-carbaldehyde (**90**) followed by oxidation to 3-formylindolyl-2-sulfonic acid (**91**). 1-Methoxy-3-aminomethyleneindole-2-thione (**92**) was found to decompose in aqueous solution (Fig **16**). The antifungal activities of brassilexin (**77**) and its metabolites indicated that brassilexin (**77**) was more antifungal to *L. maculans* than any of the products **89**-**91** ((Fig **16**, Pedras & Suchy, 2005b).

The biotransformation of brassinin (**52**) by blackleg fungus (*L. maculans*) and stem rot fungus (*S. sclerotiorum*) was investigated (Pedras & Ahiahonu, 2005, Pedras

et al., 2004). It was reported that *L. maculans* transformed brassinin (**52**) into indole-3-carboxaldehyde (**94**) and indole-3-carboxylic acid (**95**) via indole-3-methanamin (**93**, R=H) and N_b-acetyl-3-indolyl-methanamine (**93,** R=Ac) (Fig **17**). *S. sclerotiorum* metabolized brassinin (**52**) to 1-β-D-glucopyranoylbrassinin (**96**), a rather different pathway as shown in (Fig **17**).

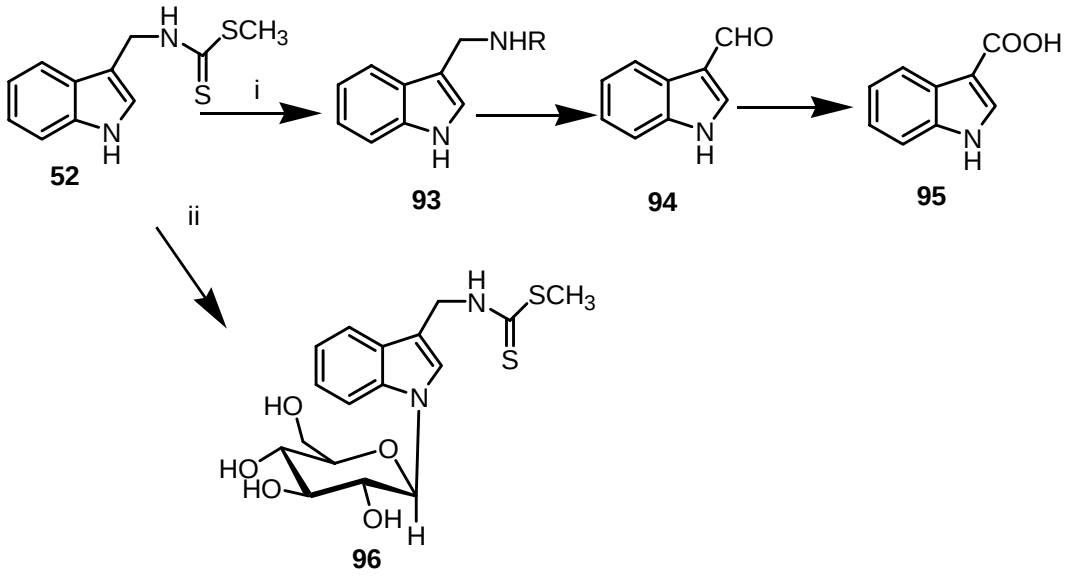


Fig **17**. Detoxification of brassinin (**58**) by i) *Leptosphaeria maculans* and ii) *Sclerotina sclerotiorum* (Pedras & Ahiahonu, 2005, Pedras *et al.*, 2004).

The biotransformation of camalexin (**78**) was investigated in mycelial cultures of *S. sclerotiorum* and *R. solani*. Camalexin (**78**)) was biotransformed by *R. solani* to two metabolites: **98** and **99** via 5-hydroxycamalexin (**97**). On the other hand *S. sclerotiorum* metabolized camalexin (**78**) to 6-oxy-(*O*-β-D-glucopyranisyl) camalexin

(**101**) via 6-hydroxycamalexin (**100**). The biotransformed products **97-101** were found to be significantly less toxic than camalexin (**78**) (Pedras & Ahiahonu, 2005, 2002).

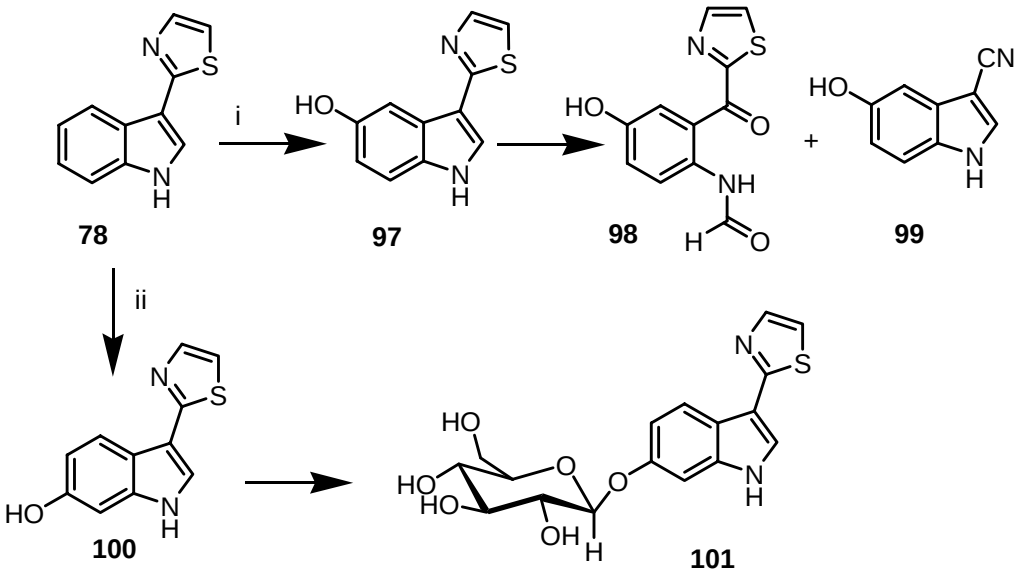


Fig **18**. Detoxification of camalexin (**78**) by i) *Rhizoctonia solani* and ii) *Sclerotinia sclerotiorum* (Pedras & Ahiahonu, 2005).

Both virulent and avirulent isolates of *L. maculans* metabolize cyclobrassinin (**62**) differently. Cyclobrassinin (**62**) was biotransformed by virulent isolates of *L. maculans* to the phytoalexin dioxybrassinin (**65**); avirulent isolates of *L. maculans* produced a different phytoalexin brassilexin (**77**), via an unstable 3-methylaminoindolyl-2-thione (**89**) (Fig **19,** Pedras & Ahiahonu, 2005).

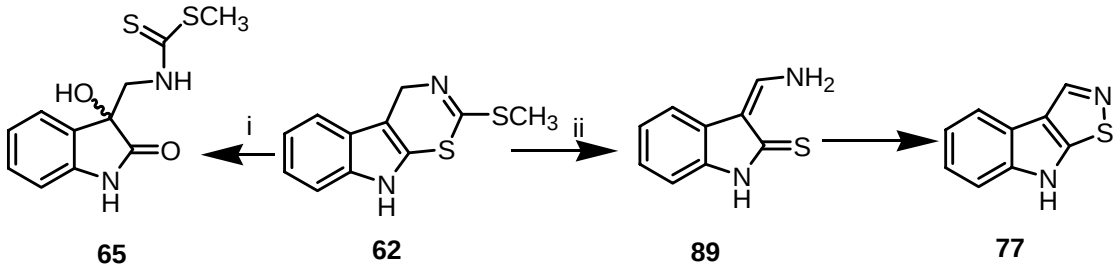


Fig **19**. Detoxification of brassinin (**58**) by i) *Leptosphaeria maculans* virulent and ii) *L. maculans* avirulent (Pedras & Ahiahonu, 2005, Pedras & Okanga, 1999)

The detoxification pathways of cyclobrassinin (**62**) by *R. solani* and *S. sclerotiorum* are different from the detoxification pathways of cyclobrassinin (**62**) by *L. maculans*. It was reported that *R. solani* transformed cyclobrassinin (**62**) into 5-hydroxybrassicanal A (**103**) via 2-sulfanylindolyl-3-carbaldehyde (**90**) and brassicanal A (**58**). *S. sclerotiorum* metabolized cyclobrassinin (**62**) to 1-β-D-glucopyranoylcyclobrassinin (**104**) (Fig **19**, **20**, Pedras *et al*., 2004a, Pedras & Ahiahonu, 2005).

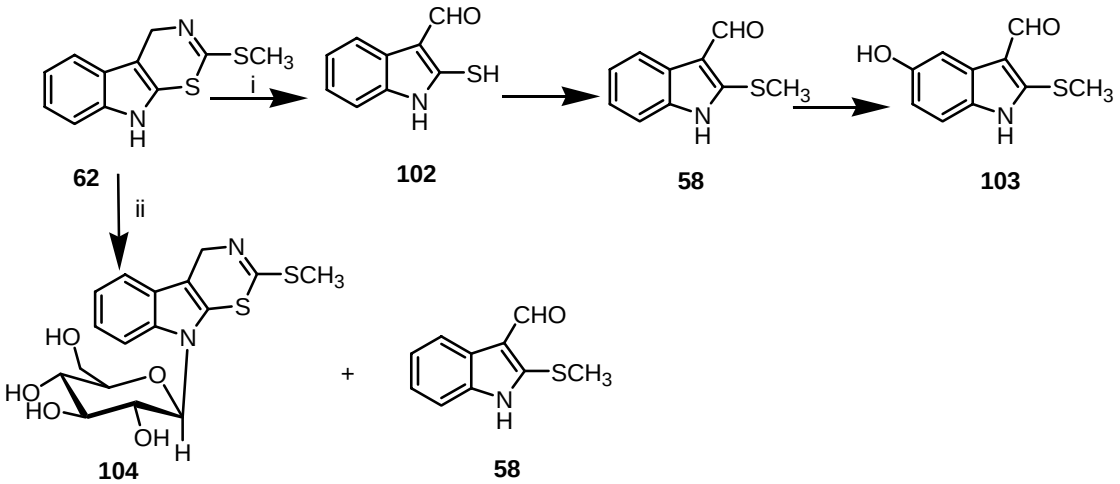


Fig **20**. Detoxification of cyclobrassinin (**62**) by i) *Rhizoctonia solani* and ii) *Sclerotina sclerotiorum* (Pedras *et al*., 2004a, Pedras & Ahiahonu, 2005)

1.3 Conclusion

Fungicides are much more active against pathogens than phytoalexins or phytoanticipins. Fungicide utilization is popular all over the world. But it is really hard to know the exact time of fungal attack to the plants and the amount of fungicide which needs to be applied. Moreover, these chemicals have adverse effects on the environment, so to save the environment from pollution, it is urgently necessary to produce disease resistant cultivars to reduce fungicide application. Understanding the defense response of both resistant and susceptible plants will assist in devising solutions to this problem.

The plant defense mechanism against fungus is very complex. To fight againt fungus plants syhthesize induced and constitutive defense compounds: phytoalexins and phytoanticipins. On the other hand fungi produce phytotoxins to invade the plants as well as detoxify the phytoalexins, produced by plants. Detoxification processes have been shown being important in the virulence of pathogens to the plans that synthesized both phytoalexins and phytoanticipins. Some wild and cultivated crucifer plants show resistance to different fungus. To understand the metabolite profiles, the aim of my project was to isolate constitutive and induced antifungal compounds to characterize and determine their structures. Synthesis of the isolated compounds to confirm the assigned structures and antifungal bioassays to determine activity were part of the research plan.

Chapter 2

2. Results and discussion

2.1. Time course experiments

Experiments were carried out to induce defense responses in plant tissues and to analyze the production of induced compounds. The induced compounds produced by cauliflower (*Brassica oleracea* var. *botrytis*), Brussels sprouts (*B. oleracea* var. *gemmifera*), broccoli (*B. oleracea* var. *italica*), Asian mustard (*B. tournefortii*), hedge mustard (*Sisymbrium officinale*), wallrocket (*Diplotaxis muralis*), Sandrocket (*D. tenufolia*) and Abyssinian mustard (*Crambe abyssinica*) were analysed over a period of time to determine the time for maximum production. The analyses were carried out using an HPLC equipped with a diode array detector and by TLC bioassay using *Cladosporium cucumerinum*. The plant tissues were treated according to the type of tissues: hard and soft, as follows.

2.1.1. Hard tissues

For the purpose of isolation of metabolites, cauliflower, Brussels sprouts, and broccoli were considered hard tissues (relative to leaf tissue). Slices were irradiated

under a UV light (250 nm wavelength) and incubated in humid and dark environment at 21-22 ºC (Pedras *et al.*, 2004d). Slices of the elicited tissues were collected at 24 hour interval up to 120 hours, ground in a blender and extracted with ethyl acetate as described in the experimental. Control (i.e. non-elicited) slices were treated in a similar manner, but without UV irradiation. Analysis of these extracts by HPLC-DAD detected the presence of fourteen peaks (R_T = 3.8, 8.8, 9.6, 11.7, 12.2, 13.9, 14.0, 16.2, 16.8, 17.8, 20.2, 24.2 and 32.8 min) in the extract of elicited cauliflower slices and eleven peaks (R_T at 4.5, 7.2, 8.7, 11.9, 13.9, 17.8, 18.0, 23.1, 28.4, 33.3 and 36.0 min) in the extract of elicited Brussels sprouts slices and eleven peaks (R_T = 4.5, 7.2, 8.7, 11.9, 13.9, 17.1, 17.8, 23.1, 28.4, 33.3 and 36.0 min) in the extract of elicited broccoli, which were absent on the chromatograms of control tissues extracts (Table **1**). The results of these time courses experiments showed that the maximum production of induced metabolites occurred around 96 hours for cauliflower and Brussels sprouts and 72 hours for broccoli (*B. oleracea* var. *italica*).

2.1.2. Soft tissues

Asian mustard, hedge mustard, wallrocket, sandrocket and Abyssinian mustard plants (3-4 weeks old) were sprayed with $CuCl_2$ and incubated at 21±2 °C temperature (Pedras *et al*., 2004c). Leaves were harvested at 24 hour intervals from both elicited and control plants. Leaves were separately frozen in liquid nitrogen, crushed, and extracted with ethyl acetate and methanol. After 12 hours, the crushed leaves were filtered and the filtrates were dried and concentrated. The residues were redissolved in acetonitrile and analysed by HPLC (Gradscr or Albpol method). Known induced compounds were identified by comparison of their UV spectra and retention

times with the phytoalexin-library available in Pedras laboratory (Pedras *et al.*, 2006). HPLC analysis indicated the presence of two compounds (R_T = 4.5 and 11.8 min) in the extract of elicited Asian mustard leaves, three compounds (R_T at 11.8 13.9 and 19.0 min) in the extract of elicited hedge mustard leaves, three compounds (R_T = 11.8, 13.9 and 22.9 min) in the extract of elicited sandrocket leaves, five compounds (R_T = 13.9, 20.1, 24.0, 28.0 and 36.1 min) in the extract of elicited wallrocket leaves and three compounds (R_T = 11.8, 13.9, 15.0 and 21.0 min) in the extract of elicited Abyssinian mustard leaves which were not detectable on the chromatograms of extracts of control leaves extracts (Table **1**). The results of these time courses experiments showed that the maximum production of these metabolites were around 72 hours for sandrocket, Abyssinian mustard and hedge mustard, 48 hours for wallrocket and 96 hours for Asian mustard leaves.

Brown mustard, an oil seed plant, was also investigated for polar metabolites. Six compounds (R_T at 7.2, 11.8, 12.2, 14.4, 14.7 and 21.0 min) were detected in the extract of elicited brown mustard leaves but not detectable on the chromatograms of extracts of control leaves extracts (Table **1**).

Table **1** Retention times for induced compounds from different species.

Obs No	Species	Retention time (minutes)
1	Abyssinian mustard	11.8, 13.9, 15.0 and 21.0 min
2	Asian mustard	4.5 and 11.8 min
3	Broccoli	4.5, 7.2, 8.7, 11.9, 13.9, 17.1, 17.8, 23.1, 28.4, 34.2 and 36.0 min
4	Brown mustard	7.2, 11.8, 14.4, 14.7 and 21.0 min (Albpol)
5	Brussels sprouts	4.5, 7.2, 8.7, 11.9, 13.9, 17.8, 18.0, 23.1, 28.4, 33.3 and 36.0 min
6	Cauliflower	3.8, 8.8, 9.6, 11.8, 12.2, 13.9, 14.0, 16.2, 16.8, 17.8, 20.2, 24.2 and 32.8 min
7	Hedge mustard	11.8, 13.9 and 19.0 min
8	Sandrocket	11.8, 13.9 and 22.9 min
9	Wallrocket	13.9, 20.1, 24.0, 28.0 and 36.1 min

2.2. Cauliflower (*Brassica oleracea* var. *botrytis*)

Using HPLC analysis of elicited tissues, seven induced compounds were detected in cauliflower florets (*B. oleracea* var. *botrytis*). In order to isolate these metabolites to determine their structures, larger scale experiments were performed as described below.

2.2.1. Isolation of elicited compounds

In order to isolate elicited metabolites, which were potentially new phytoalexins, large scale experiments were carried out with cauliflower florets which were irradiated with a UV light. Elicited slices were extracted with ethyl acetate and the solvent was evaporated. The residue was fractionated by gradient flash column chromatography (C_6H_{12}:CH_2Cl_2:MeOH) as described in the experimental. Fractions F 6, F 8, F 10 and F 11 were further subjected to multiple chromatographies guided by HPLC analysis to isolate the elicited compounds with retention times at R_T = 17.8, 16.8, 16.2, 12.2, 9.6, 8.8 and 3.8 min respectively (Fig **21,** Fig **22**). The last four compounds were isolated from F11 (Fig **21**).

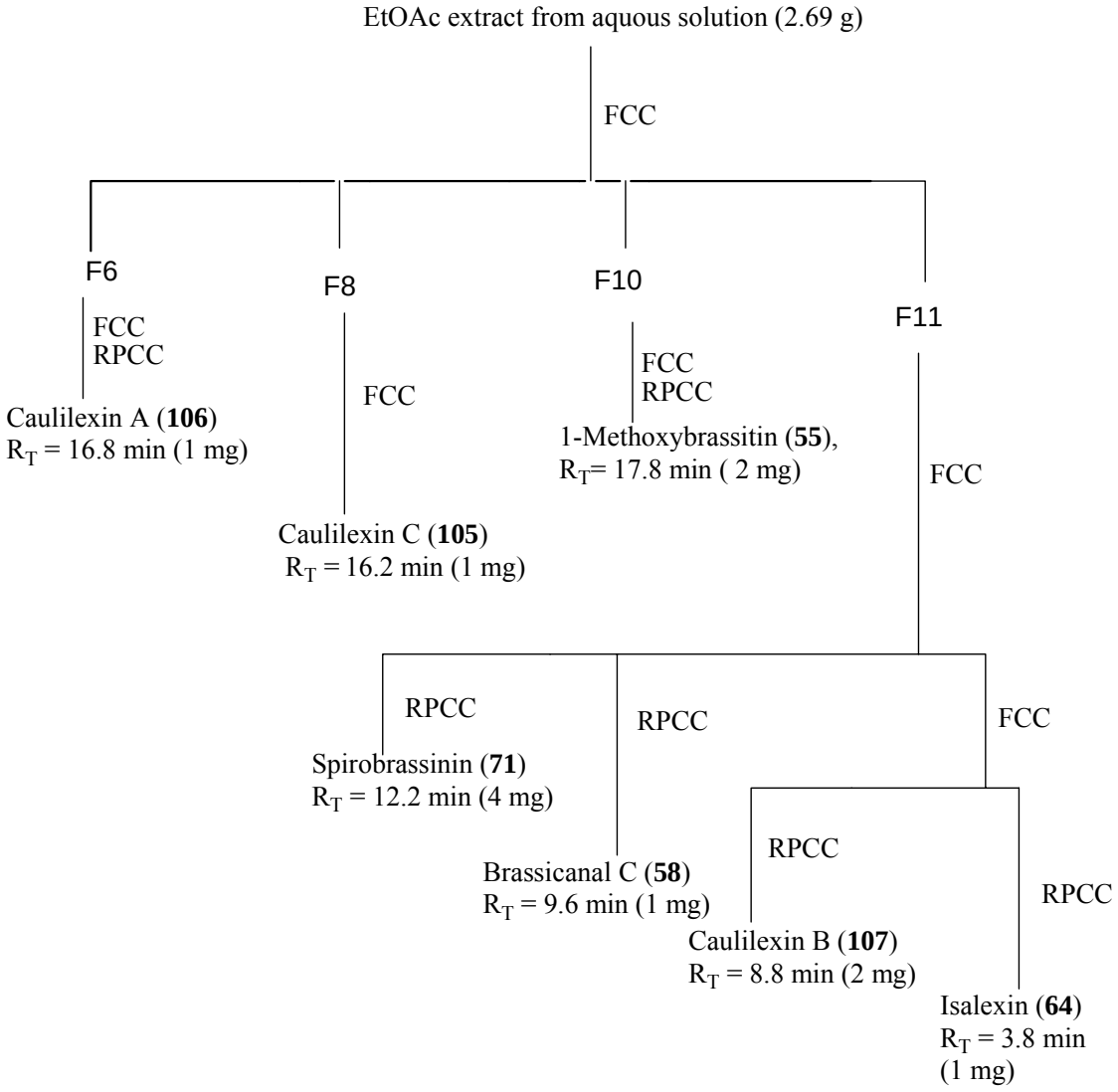


Fig **21**. Isolation of compounds from crude extract of cauliflower (*Brassica oleracea* var. *botrytis*)

The most polar component (R_T = 3.8 min) in the EtOAc extract had the molecular formula $C_9H_7NO_3$, as determined by HREIMS. The UV and ^{1}H-NMR spectral data were identical with those of isalexin (**64).** Isalexin was first isolated as a phytoalexin from rutabaga tubers (Pedras *et al*., 2004f).

The component with R_T = 9.6 min was isolated by reversed phase column chromatography; HRMS indicated that the molecular formula of this compound was $C_{10}H_9NSO_3$. The UV spectra and ^{1}H-NMR spectral data were identical with those of brassicanal C (**60**). The optical rotation of this compound was not determined, due to the small amounts isolated. Brassicanal C (**60**) was first isolated as a phytoalexin from cabbage (Monde *et al.*, 1991) and synthesized recently (Pedras *et al.*, 2006b).

The component with R_T = 12.2 min was isolated by flash column chromatography and HREIMS indicated that the molecular formula of this compound was $C_{11}H_{10}N_2S_2O$. The UV and ^{1}H-NMR spectral data were identical with those of spirobrassinin (**71**). The optical rotation of spirobrassinin was $[\alpha]_D^{24}$ -109 (CH_2Cl_2, c 0.35) and its absolute configuration was determined to be *S* (analysed by chiral solvation, Pedras *et al*., 2004d). Spirobrassinin (**71**) was first isolated as a phytoalexin from *Raphanus sativus* L. var. *hortensis.* (Takasugi *et al*., 1987).

The component with R_T = 17.8 min was isolated by reverse phase column chromatography (CH_3CN:H_2O, 4:6). HRMS indicated that the molecular formula of this compound was $C_{12}H_{14}N_2SO_2$. The UV and ^{1}H-NMR spectral data was identical to those of 1-methoxybrassitin (**55**). 1-Methoxybrassitin (**55**) was first isolated as a phytoalexin from Chinese cabbage inoculated with *Pseudomonas cichorii.* (Takasugi *et al.*, 1988).

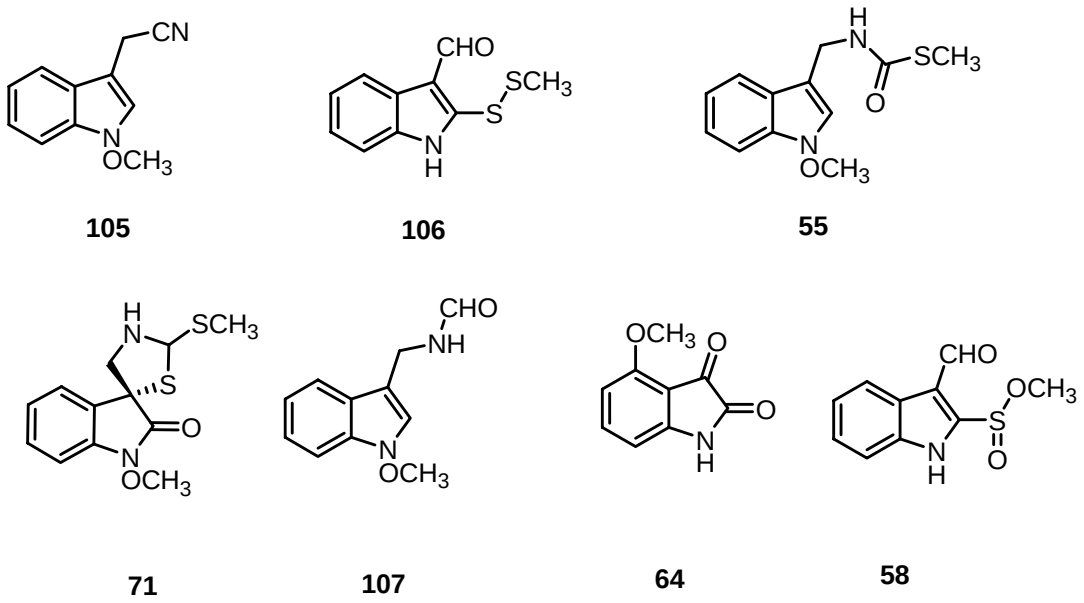


Fig **22**. Phytoalexins from cauliflower: caulilexin C (**105**), caulilexin A (**106**), 1-methoxinbrassinin (**55**), spirobrassinin (**71**), caulilexin B (**107**), isalexin (**64**), and brassicanal C (**58**).

The compound with R_T = 16.2 min (**106**) was found to have the molecular formula $C_{10}H_9NOS_2$, indicating seven degrees of unsaturation (HRMS). In the ^{1}H-NMR spectrum, the presence of four aromatic protons suggested that the compound was 2,3-disubstitued indole containing an aldehydic proton at δ_H 10.20 (s, 1H) and a methylsulfanyl group at δ_H 2.60 (s, 3H) respectively. The ^{13}C-NMR spectrum confirmed the presence of 10 carbons. The corresponding chemical shifts at δ_C 184.4 and δ_C 23.6 suggest an aldehydic and a methylsulfanyl group respectively. Thus, the proposed structure was 2-dithiomethoxyindole-3-carboxaldehyde (**106**) and this structure was confirmed by synthesis (Pedras *et al*., 2006a). The compound was given the name caulilexin A, by analogy with a related phytoalexin, brassicanal A (**58**). This compound is the first indole phytoalexin containing a disulfide bridge.

The compound with R_T = 16.8 min was found to have the molecular formula $C_{11}H_{10}N_2O$ with eight degrees of unsaturation (HRMS). The presence of five aromatic protons suggested that the structure contained a 2- or 3-substituted indole nucleus. Signals in the ^{1}H-NMR spectrum at δ_H 4.24 (3H) and δ_H 3.91 (2H) and ^{13}C-NMR at δ_C 67.5 and 14.5 confirmed the presence of methoxy and methylene groups. In the FTIR spectrum, the absorption at 2249 cm^{-1} indicated the presence of a nitrile group (δ_C 119.6 in ^{13}C-NMR) and the structure was assigned as 1-methoxyindol-3-ylacetonitrile (**105**). The compound was given the name caulilexin C. The proposed structure was confirmed by synthesis, as described later in this study. Caulilexin C (**105**) has been isolated as a constitutive metabolite from Chinese cabbage (Nomoto *et al.*, 1970). Incubation of indole glucosinolate with myrosinase also led to the formation of 1-methoxyindole-3-yl acetonitrile (**105**) (Agerbirk *et al.*1998, Hanley *et al.*1990). Caulilexin C (**105**) was isolated as induced metabolite for the first time in this work.

The molecular formula of the compound with retention time R_T = 8.8 min was established as $C_{11}H_{12}N_2O_2$ having seven degrees of unsaturation (HREIMS). In the ^{1}H-NMR spectrum, five aromatic protons were observed, suggesting that the structure contained a 2- or 3-substituted indole. Signals in the ^{1}H-NMR spectrum at δ_H 4.07 (s, 3H) and δ_H 4.52 (d, J = 6 Hz, 2H) and in the ^{13}C-NMR spectrum at δ_C 67.0 (q) and δ 33.7 (t) respectively, indicated the presence of methoxy and methylene protons. The singlet at δ 8.14 (1H, s) indicated the presence of a formamide group. Consequently, the structure was assigned 1-methoxyindol-3-ylmethylformamide. The proposed structure was confirmed by synthesis as described later. 1-Methoxyindol-3-ylmethylformamide (**107**) was likely derived from the degradation of brassinin and was

given the name caulilexin B. Caulilexin B represents a new example of a non-sulfur indole phytoalexin.

Table **2**. Metabolites[a] of phytoalexins[b] in cauliflower florets (*Brassica oleraceae* var. *botrytis*) upon elicitation with UV light (250 nm).

Metabolite	48 hours	72 hours	96 hours	120 hours
Caulilexin A **(106)**	0.59 ± 0.04	7.8 ± 0.2	1.7 ± 0.1	1.3 ± 0.8
Caulilexin B **(107)**	0.84 ± 0.02	1.8 ± 0.2	2.3 ± 0.5	0.64 ± 0.09
Caulilexin C **(105)**	0.12 ± 0.01	0.55 ± 0.13	0.39 ± 0.08	0.20 ± 0.01
Isalexin **(64)**	0.062 ± 0.014	0.23 ± 0.03	0.49 ± 0.05	0.46 ± 0.06
S-(-)-Spirobrassinin **(71)**	0.51 ± 0.07	24.4 ± 6.5	39.7 ± 1.8	11.1 ± 2.5
1-Methoxybrassitin **(55)**	17.0 ± 0.22	0.75 ± 0.01	0.54 ± 0.06	0.30 ± 0.0
Brassicanal C **(60)**	2.2 ± 0. 5	4.9 ± 0.9	0.43 ± 0.09	3.0 ± 0.9
Cyclobrassinin **(62)**	0.025±0.002	0.079±0.006	0.50±0.00	0.088±0.06
1-Methoxy Spirobrassinin **(72)**	0.049±0.007	0.057±0.008	0.030±0.008	0.012±0.002
Sinalexin **(83)**	0.007±0.001	0.07±0.01	0.028±0.002	0.17±0.09

[a] Amounts of phytoalexins are μmol/g tissues.
[b] Results are presented as mean ± standard deviation.

2.2.3. Syntheses of elicited compounds

Caulilexin C **(105)** (1-methoxyindolyl-3-acetonitrile) was synthesized from tryptamine (**108**) as shown in Fig **23.** The key intermediate 1-methoxyindole-3-acetaldoxime (**88**) was synthesized from tryptamine (**108**) by protecting the amino

group with acetyl followed by reduction, oxidation and methylation using sodium cyanoborohydride, hydrogen peroxide and dimethylsulfate to yield 1-methoxy-N_b-acetyltryptamine (**111**) (Pedras & Okinyo, 2006a). Hydrolysis of compound **111** with methanolic sodium hydroxide yielded the corresponding amine, which was oxidized with hydrogen peroxide to yield 1-methoxyindole-3-acetaldoxime (**88,** Pedras *et al.,* 2004f). Upon treatment with acetic anhydride, 1-methoxyindole-3-acetaldoxime (**88**) yielded caulilexin C (**105**) in 8% overall yield.

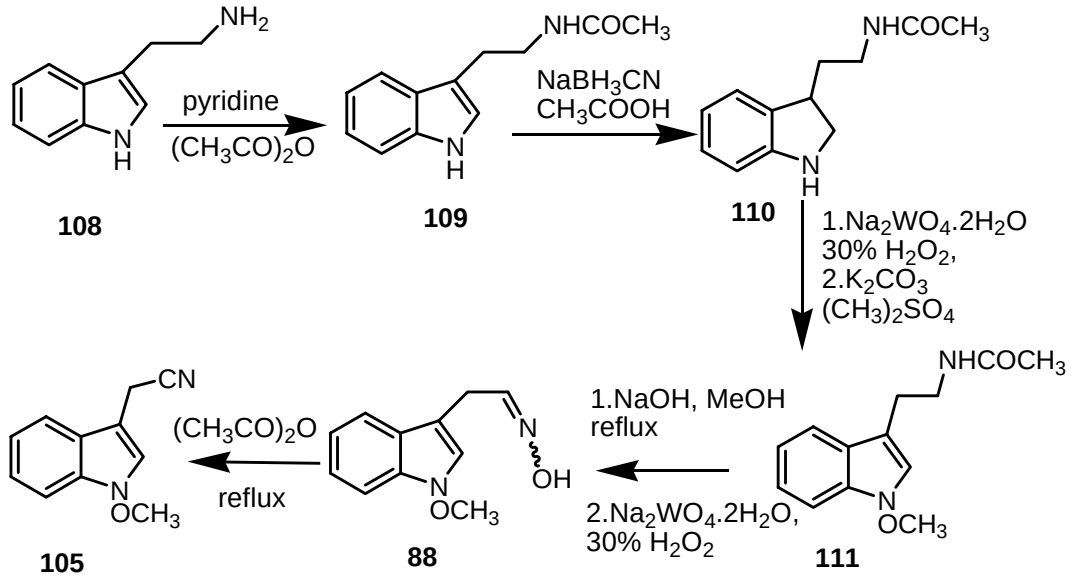
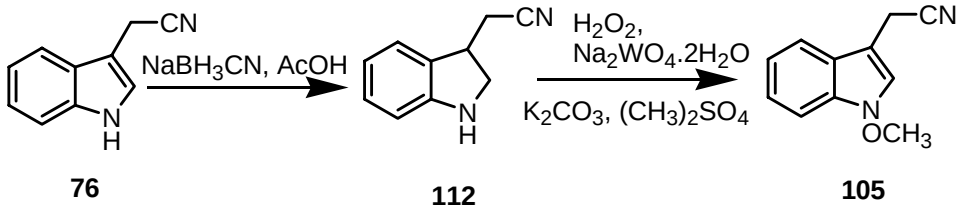


Fig **23**. Synthesis of caulilexin C (**105,** overall yield 10%).

Caulilexin C (**105**) was also synthesized by another route. The reduction of commercially available indolyl-3-acetonitrile (**76**) with $NaBH_3CN$-AcOH yielded 2,3-dihydrogenindolyl-3-acetonitrile (**112) (**Gribble 1998), which was oxidized with H_2O_2-Na_2WO_4-mediate oxidation of **112,** followed by methylation with $(CH_3)_2SO_4$ to yield

caulilexin C (**105**) in 10% overall yield (Fig **23, 24**). Although these reactions gave caulilexin C (**105**) in low yield, both routes are more efficient than the previous syntheses. (Acheson *et al.,* 1984, Somei *et al*., 1985).



76 **112** **105**

Fig **24**. Synthesis of caulilexin C (**105,** overall yield 10%).

The synthesis of caulilexin B was accomplished in five steps from indoline (**113**) via 1-methoxyindole (**114**) and oxime (**116**) as outlined in Fig **25**. The intermediate 1-methoxyindole (**114**) was prepared from indoline by oxidation and methylation using hydrogen peroxide and dimethylsulfate. Thus 1-methoxyindole was formylated under Vilsmeier condition (Smith, 1954). The resulting 1-methoxyindole-3-carboxaldehyde (**115**) quantitatively yielded to 1-methoxyindole-3-carboxaldehyde oximes (**116**) upon treatment with hydroxylamine hydrochloride in ethanol. The intermediate 1-methoxyindole-3-acetaldoxime (**88**) was reduced with sodium borohydride in the presence of $NiCl_2.6H_2O$ to the corresponding 1-methoxy-indole-3-yl methyl amine (Pedras *et al.*, 2003) and followed by formylation under in reflux with ethyl formate to yield caulilexin B (**107**) with 17% overall yield (Fig **25**).

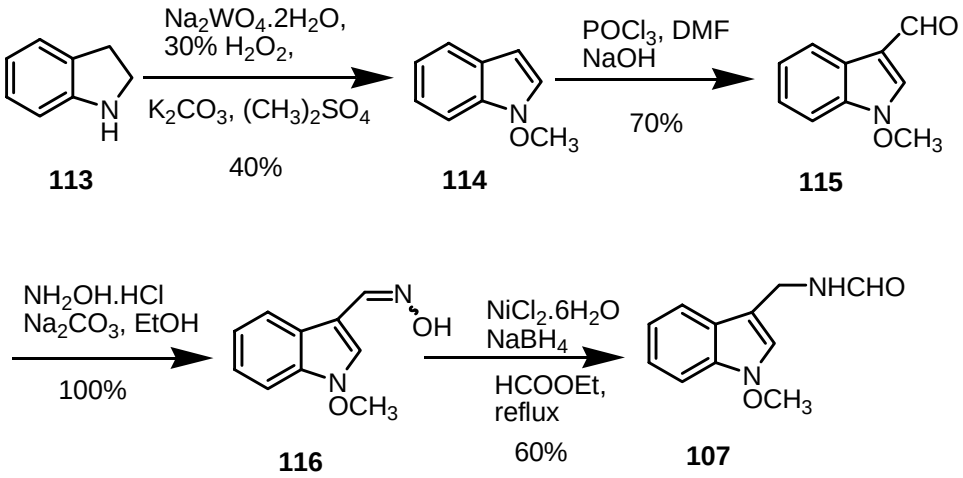


Fig **25**. Synthesis of caulilexin B (**107**)

2.2.4. Bioassay of elicited compounds

Antifungal activities of caulilexins A-C (**106, 107** and **105**) against fungal pathogens (*L. maculans/Phoma lingam, S. sclerotiorum* and *R. solani)* of cruciferous plants were established using radial growth antifungal bioassays. Antifungal activities of caulilexins A-C (**106, 107** and **105**) against *C. cucumerinum* were determined using a TLC antifungal bioassay. Activities of caulilexin A (**106**) and caulilexin C (**105**) were compared with those of structurally related phytoalexins, brassicanal A (**58**) and arvilexin (**84**). The results of these studies are shown in Table 2. Caulilexin A (**106**) appears to exhibit the strongest antifungal activity against *R. solani* and *S. sclerotiorum*. It causes complete inhibition of the growth of *R. solani* at 5.0×10^{-4} M (twice as antifungal as brassicanal A (**58**), whereas the growth of *S. sclerotiorum* is completely inhibited even at 1.0×10^{-4} M (100 times more antifungal than brassicanal

A (**58**). Moderate antifungal activity of caulilexin A (**106**) 55% (5.0×10^{-4} M) against *L. maculans / P. lingam* is slightly lower than the antifungal activity of brassicanal A (Pedras *et al*., 2006b). Caulilexin C (**105**) appears to be slightly more antifungal than arvilexin (**84**) and caused complete inhibition (5.0×10^{-4} M) of the fungal growth in *R. solani* and slightly lower antifungal activity (77%) against *L. maculans / P. lingam*. Caulilexin B (**107**) exhibited the lowest antifungal activity among the new phytoalexins. In a TLC bioassay against *C. cucumerinum*, caulilexin A (**106**) caused inhibition of growth at 1.0×10^{-8} M, whereas caulilexin B and C (**105** and **107**) caused inhibition of the growth at 1.0×10^{-6} M.

Table **3**. Antifungal activity [a] (% of inhibition) of caulilexins A, B, C (**106**,**107**,**105**) , brassicanals A (**58**) and C (**60**), and arvelexin against *Leptosphaeria maculans*, *Sclerotinia sclerotiorum* and *Rhizoctonia solani.*

Metabolite	Conc. (M)	*L. maculans /Phoma lingam* [b]	*Sclerotinia sclerotiorum* [c]	*Rhizoctonia solani* [d]
Caulilexin A	5.0×10^{-4}	55 ± 7	100 ± 0	100 ± 0
(106)	2.5×10^{-4}	39 ± 3	100 ± 0	83 ± 9
	1.0×10^{-4}	21 ± 7	100 ± 0	50 ± 8
	5.0×10^{-5}	10 ± 6	90 ± 7	13 ± 2
	1.0×10^{-5}	n.d.	71 ± 12	n.d.
Brassicanal A	5.0×10^{-4}	70 ± 5	33 ± 9	53 ± 4
(58)	2.5×10^{-4}	62 ± 7	12 ± 5	33 ± 7
	1.0×10^{-4}	30 ± 5	8 ± 2	n. i
	5.0×10^{-5}	13 ± 4	n. i.	n. i..
Brassicanal C	5.0×10^{-4}	70 ± 5	53 ± 4	33 ± 9
(60)	2.5×10^{-4}	62 ± 5	33 ± 7	11 ± 4
	1.0×10^{-4}	30 ± 5	n. i.	n. i.
	5.0×10^{-5}	10 ± 4	n. i.	n. i.
Caulilexin B	5.0×10^{-4}	31 ± 8	31 ± 1	18 ± 7
(107)	2.5×10^{-4}	17 ± 6	20 ± 2	n. i.
	1.0×10^{-4}	n. i.	n. i.	n. i.
Caulilexin C	5.0×10^{-4}	77 ± 2	30 ± 8	100 ± 0
(105)	2.5×10^{-4}	45 ± 5	n. i.	80 ± 7
	1.0×10^{-4}	30 ± 10	n. i.	48 ± 11
Arvilexin	1.0×10^{-4}	59 ± 3	37 ± 3	70 ± 3
(84)	2.5×10^{-4}	36 ± 3	17 ± 3	17 ± 7
	5.0×10^{-4}	15 ± 2	n. i.	n. i.

[a] % Inhibition = 100 - [(growth on treated/growth on control) × 100] ± standard deviation; results are the means of at least three separate experiments; n.i., no inhibition; n.d., not determined.
[b] Results after 96 hours of incubation.
[c] Results after 48 hours of incubation.
[d] Results after 72 hours of incubation.

2.3. Brussels sprouts (*Brassica oleracea* var. *gemmifera*)

In time course experiments, nine induced metabolites were detected in extracts of Brussels sprouts (*B. oleracea* var. *gemmifera*) of which five are known and four were unknown. To isolate these metabolites, large scale experiments were carried out.

2.3.1. Isolation of metabolites

To isolate the elicited metabolites, large scale experiments were carried out with Brussels sprouts irradiated with UV light. Elicited slices or leaves were ground and extracted with EtOAc. The solvent was evaporated under reduced pressure and the residue was fractionated by gradient flash column chromatography (C_6H_{12}:CH_2Cl_2:MeOH), as described in the experimental. Fractions F 6, F 7, F 8, F 11, F 12 and F 13 were further subjected to multiple chromatography to yield the elicited compounds with (HPLC) retention time R_T = 4.5, 7.2, 8.7, 11.9, 13.9, 17.8, 18.0, 23.1, 28.4, 33.3 and 36.0 min (Fig **26,** Fig **27**).

The molecular formula of the elicited compound with R_T = 33.3 min was determined to be $C_{19}H_{18}N_2O_2$ by HRMS, which suggested twelve degrees of unsaturation. The ^{1}H-NMR (CD_3CN) spectrum of this metabolite showed ten aromatic protons as well as two methylene protons at δ_H 4.17 and six methoxy protons at δ_H 4.03 (s). Therefore, di-(1-methoxy-3-indolyl)methane (**120**) was assigned to this elicited compound with R_T = 34.6 min. The proposed structure was confirmed by synthesis, as

described later in this study. Di-(1-methoxy-3-indolyl)methane (**120**) was previously obtained in the neoascorbigen synthesis (Yudina *et al.*, 2000).

The molecular formula of the elicited compound with R_T = 28.4 min was determined to be $C_{18}H_{16}ON_2$ by HRMS, which suggested twelve degrees of unsaturation. The ^{1}H-NMR (CD_3CN) spectrum of this metabolite showed ten aromatic protons of indole ring, as well as three methoxy protons at δ_H 4.03 (s), two methylene protons at δ_H 4.20 (s) and one NH proton at δ_H 9.01 (s). Therefore, this elicited compound with R_T = 28.4 min was assigned as 1-methoxy-3,3'-diindolylmethane (**119**). The proposed structure was confirmed by synthesis, as described later in this study.

The molecular formula of the elicited compound having R_T = 23.1 min was determined to be $C_{17}H_{14}N_2$ by HRMS, which suggested twelve degrees of unsaturation. The ^{1}H-NMR (in CD_3CN) spectrum of this metabolite showed ten aromatic protons as well as two methylene protons at δ_H 4.22 (s) and two NH protons at δ_H 9.03 (bs). Two C-2 protons were at δ_H 7.08 (s), indicated that two indole moiety had the same substitution. Therefore, diindolylmethane (**50)** was assigned to this elicited compound with R_T = 23.1 min. The proposed structure was confirmed by synthesis as described later in this study.

Table **4** Production[a] of induced metabolites in Brussels sprouts (*Brassica oleraceae* var. *gemmifera*) upon elicitation with UV light (250 nm).

Metabolite	48 h	72 h	96 h	120 h
Brussalexin A **(121)**	0.15±0.05	1.07±0.012	0.59 ± 0.01	0.39±0.04
Brussalexin B **(117)**	0.02±0.00	0.80±0.12	0.34±0.02	0.022±0.004
Brussalexin C **(118)**	0.10±0.00	0.44±0.14	0.19±0.00	0.004±0.003
1-Methoxy-3, 3'-diindolylmethane **(119)**	0.081±0.00	0.245±0.00	0.14±0.01	0.006±0.003
Di-(1-methoxy-3-indolyl)methane **(120)**	0.05±0.01	0.19±0.03	0.12±0.02	0.028±0.01
Diindolylmethane **(50)**	0.034±0.0	0.13±0.01	0.08±0.02	0.0029±0.00
1-Methoxybrassitin **(55)**	-	1.17	-	-
Ascorbigen **(51)**	-	4.21	-	-

[a]Amounts of induced metabolites are ηmol/g tissues
[b]Results are presented as mean ± standard deviation.

The HRMS spectrum of the compound having R_T = 7.0 min indicated the molecular formula $C_{10}H_{11}O_2SN$, (*m/z* 209.0509). The ^{1}H-NMR (CD_3CN) spectrum of this metabolite showed five aromatic protons attributable to an indole, as well as two methylene protons at δ_H 4.50 (2H, s), three thiomethyl protons at δ_H 2.80 (3H, s) and one NH proton at δ_H 9.53 (1H, bs, D_2O exchangeable). ^{13}C-NMR (CD_3CN) spectrum peaks at δ_C 52.2 and 38.9 indicated methylene group and methyl group attached to sulfoxide. Therefore, 3-methanesulfonylmethylindole (**117**) was assigned to this

elicited compound with R_T = 7.2 min. The compound was given the name brussalexin B (**117**). To confirm this structure, brussalexin B (**117**) was synthesized as shown later. The spectral data of the isolated compound were identical with those of the synthesized compounds.

The molecular formula of the elicited compound having R_T = 8.7 min was determined to be $C_{11}H_{13}SNO_3$ by HRMS-EI (*m/z* 239.0617), which suggested seven degrees of unsaturation. The ^{1}H-NMR spectrum of this metabolite showed four aromatic protons as well as two methylene and three methoxy protons. The spin system of aromatic protons showed that the proton at δ_H 7.11 (H-6, dd, *J* = 7.5, 7.5 Hz) was coupled to two protons at δ_H 7.08 (H-7, d, *J* = 7.5 Hz) and δ_H 6.61 (H-5, d, *J* = 7.5 Hz), the coupling constant further suggested that the orientation of H-6 to H-7 and to H-5 is *ortho.* A D_2O exchangeable broad proton δ_H 9.50 and a doublet at δ_H 7.27 (d, *J* = 2.5 Hz, with N-H proton), suggested the aromatic protons to be part of indole ring having substitution at either C-4 or C-7. HMBC spectrum analysis showed correlations of a downfield carbon (C-4) at δ_C154.6 with methoxy protons, and the methylene protons at δ_H 4.64 with carbons at δ_C 102.8 (C-2), δ_C 116.9 (C-4a) and δ_C 125.6 (C- 3). The signals at δ_C 55.0, 53.2 and 38.9 indicated the carbons of methoxy, methylene and methyl attached with sulfone groups. Therefore, the structure of this elicited metabolite was assigned as methyl 4-methoxy-3-methylsulfanylmethylindol. The compound was given the name brussalexin C (**118**). The structure of brussalexin C (**118**) was confirmed by synthesis, as shown later in this study.

The HRMS of the component having R_T = 17.8 min, indicated that the molecular formula of this compound is $C_{12}H_{14}N_2SO_2$. The UV spectra and NMR

signals were identical with those of authentic 1-methoxybrassitin (**55**) sample. 1-Methoxybrassitin (**55**) was also previously isolated from cauliflower (Pedras *et al.*, 2006) and described previously in this chapter.

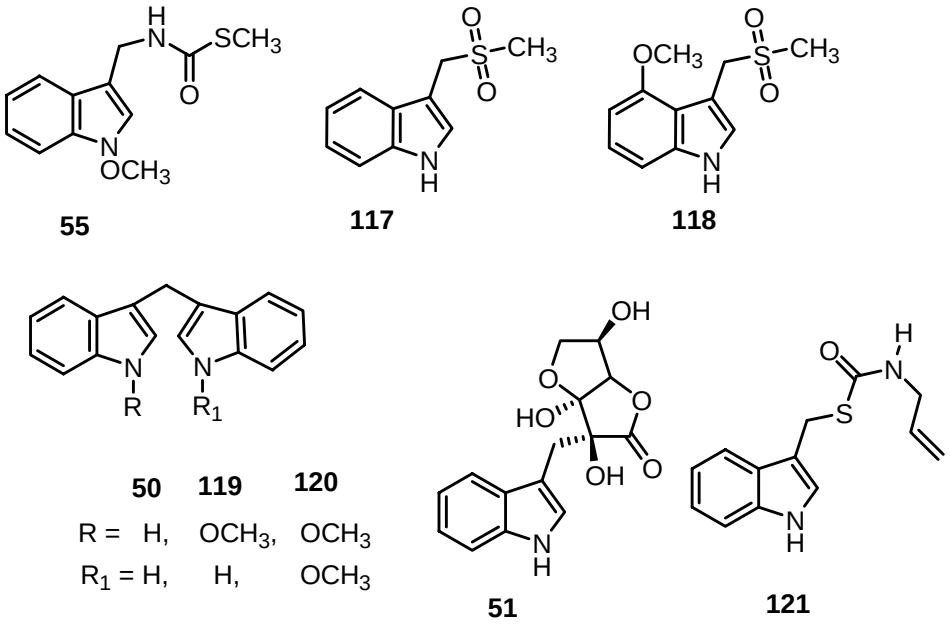


Fig **26**. Phytoalexins 1-methoxybrassitin(**55**), brussalexin A (**121**), B (**117**) and C (**118**), and compounds diindolylmethane (**50**), 1-methoxy-3, 3'-diindolylmethane (**119**) and di-(1-methoxy-3-indolyl)methane (**120**) and ascorbigen (**51**) from Brussels sprouts.

The HRMS spectrum of the compound having R_T = 18.0 min indicated the molecular formula $C_{13}H_{14}N_2SO$. The ^{1}H-NMR (CD_3CN) spectrum of this metabolite showed five aromatic protons, as well as four methylene (δ_H 4.34, s, 2H and δ_H 3.85, m, 2H), and three olifinic protons at δ_H 5.81 (*m*), 5.06 (dd, *J* = 11, 2 Hz) and 5.17 (dd, *J* = 14, 2 Hz). The spin system of aromatic protons showed that the proton at δ_H 6.96

(H-5, dd, J = 7, 8 Hz) was coupled to two protons at δ_H 7.61 (H-4, d, J = 8 Hz) and δ_H 7.05 (H-6, d, J = 7, 8 Hz); the coupling constant further suggested that the proton at δ_H 7.05 (H-6, dd, J = 7, 8 Hz) was coupled to two protons at δ_H 6.96 (H-5, dd, J = 7, 8 Hz) and 7.41 (H-7, d, J = 8 Hz). One D_2O exchangeable broad proton δ_H 9.10 for NH and a doublet at δ_H 7.23 (d, J = 2.5 Hz, with N-H proton) for H-2 proton, suggested the aromatic protons to be part of an indole ring having substitution at C-3. Terminal vinyl proton δ_H 5.17 (dd, 1H, J = 17, 2.5 Hz) was coupled with geminal proton δ_H 5.17 (dd, 1H, J = 11, 2.5 Hz) and trans proton δ_H 5.81 (m, 1H). The remaining atoms (HCNOS) required to fulfill the molecular formula were used for connecting the methylene with allyl group. Further more the allyl group showed coupling with an exchangeable proton, Therefore, [S-(indol-3-yl) methylallylthiocarbamate] (**121**) was assigned as the possible structure of this elicited metabolite with R_T = 18.0 min (Pedras *et al*., 2007a). The compound was given the name brussalexin A (**121**). The structure was confirmed by synthesis, as shown later. The spectral data were identical with those of a synthesized sample.

The HREIMS spectrum of the compound at R_T = 4.5 min indicated the molecular formula $C_{13}H_{14}N_2SO$. The ^{1}H-NMR (CD_3CN) spectrum of this metabolite showed five aromatic protons, along with one NH proton (D_2O exchangeable). This indicated an indole ring with substitution at C-3 position. Based on ^{1}H-NMR and ^{13}C-NMR signals the structure was assaigned as ascorbigen (**51**) and the spectral data were similar to published work (Lazhko *et al*., 1993). The optical rotation was $[\alpha]_D^{24}$ -30 in CH_2Cl_2 at c 0.50. Ascorbigen (**51**) was first isolated from Savoy cabbage (Prochazka &

Zelimir,1958, Prochazka & Severa., 1960). Kutacek group found that ascorbigen (**51**) was biogenenatically synthesized from the precursor tryptophan (Kutacek *et al.,* 1960).

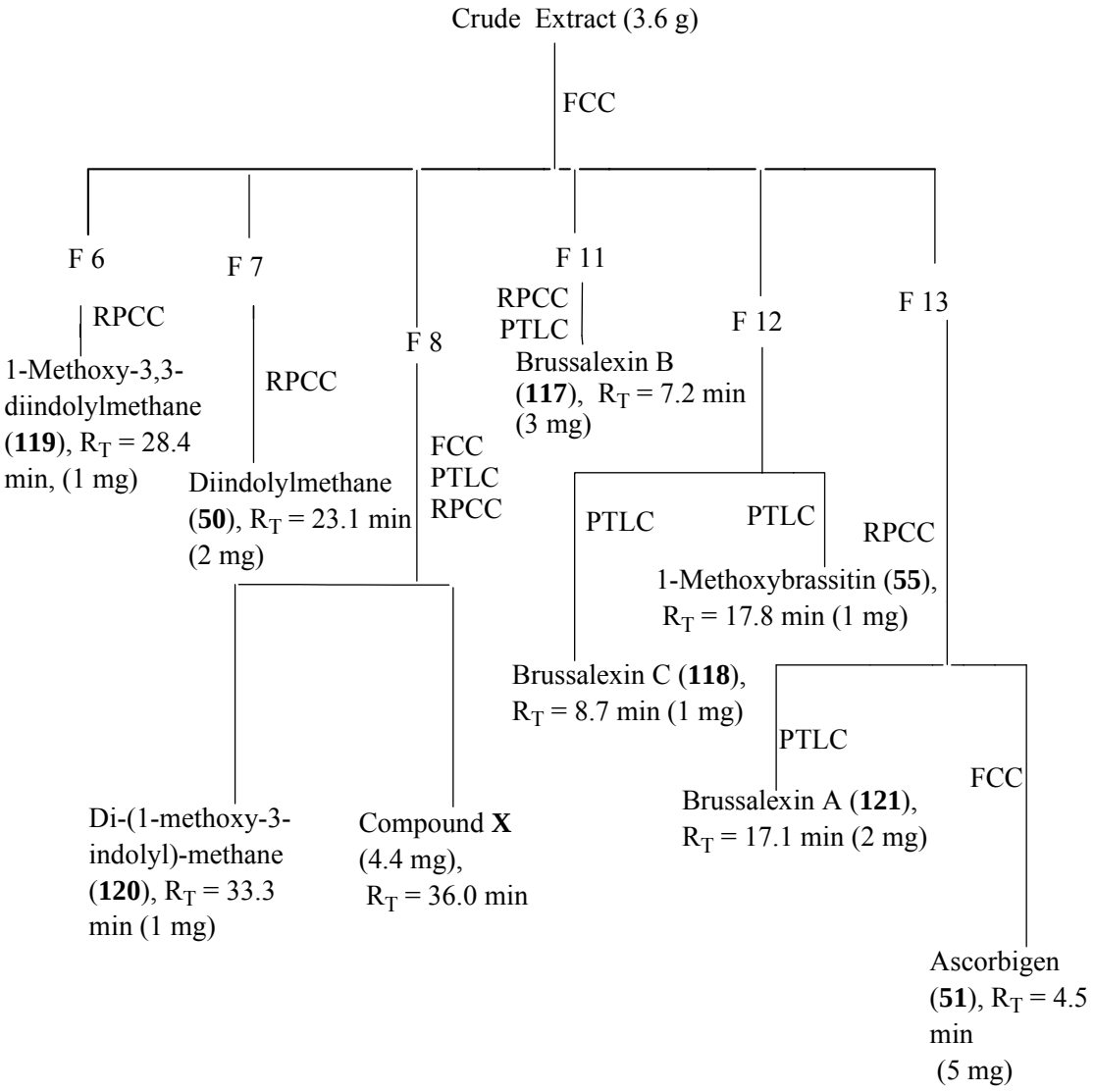


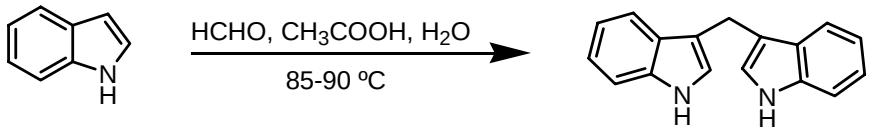
Fig **27**. Isolation of compounds from crude extract of Brussels sprouts

The compound **X** with R_T = 36.0 min was isolated through flash column chromatography and reverse phase column chromatography (Fig **27**). The color of this

compound is white in solid but brown in solution (CH_2Cl_2). High resolution mass spectrometry of the compound **X** indicated the likely molecular ion *m/z* 371 but did not indicate the molecular formula. The ^{1}H-NMR (in CD_3CN) spectrum of this metabolite indicated seventeen aromatic protons and three NH (δ_H 9.06, bs, 1H and 8.82, bs, 2H) D_2O exchangeable protons (probably two indole ring). The ^{13}C-NMR spectrum indicated the presence of eighteen peaks between 150-99 ppm. The UV spectrum and retention time of compound **X** did not match with any of the previously isolated crucifer phytoalexins available in Pedras' library (Pedras *et al.,* 2006a and unpblished works in Pedras lab).

2.3.2. Synthesis of metabolites

The synthesis of diindolylmethane (**50**) was carried out, as shown in Fig **28.** Diindolylmethane (**50**) was synthesized by the reaction of indole (**122**) with formaldehyde at 90°C (90% yield) (Jackson *et al.,* 1987).



122 **50**

Fig **28**. Synthesis of diindolylmethane (**50**).

To synthesize 1-methoxy-3,3'-diindolylmethane (**119**), the key intermediate 1-methoxyindole-3-methanol (**123**) was prepared from 1-methoxyindole (**114**) by Vilsmeier formylation under Vilsmeier conditions (Smith, 1954) followed by reduction. Reaction of 1-methoxyindole-3-methanol (**123**) with indole in presence of acetic acid at 50°C, yielded 1-methoxy-3,3'-diindolylmethane (**119,** overall yield 34%, Fig **29**).

Reaction of 1-methoxyindole (**114**) with 40% formaldehyde gave product di-(1-methoxy-3-indolyl)-methane (**120,** 25% yield). Although the overall yield of di-(1-methoxy-3-indolyl)methane (**120**) obtained by this method was low (25% yield), previous syntheses appear to be less efficient (<2% yield, Fig **29**,Yudina *et al.*, 2000).

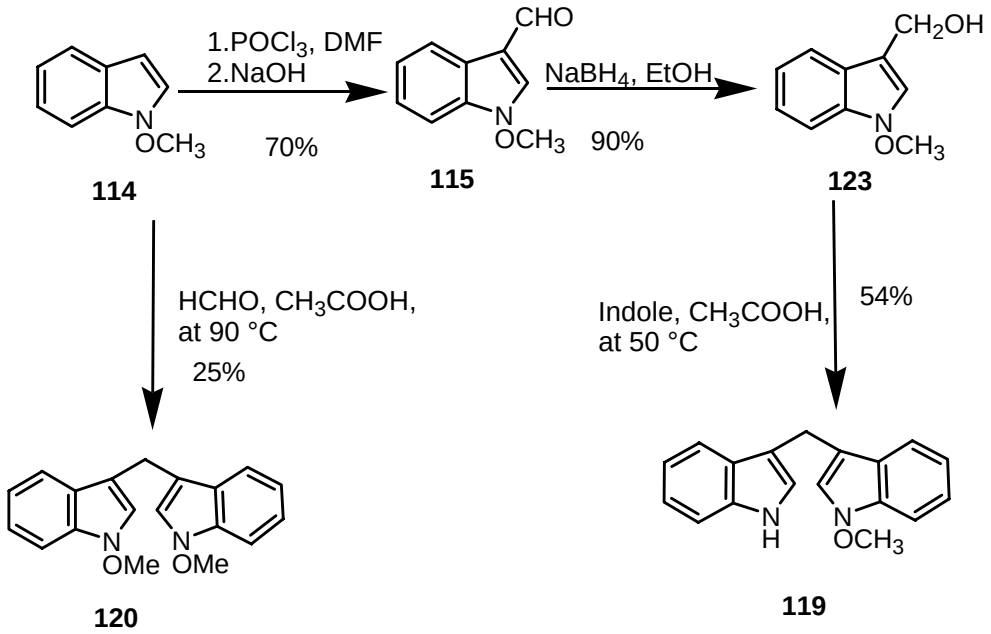


Fig **29**. Synthesis of 1-methoxy-3,3'-diindolylmethane (**119**) and di-(1-methoxy-3-indolyl)methane (**120)**.

Brussalexin B (**117**) was prepared from indole-3-methanol (**49,** Fig **30**). Indole-3-methanol was treated with Et_3N and $SOCl_2$ at 0°C for 40 min to form unstable indolyl-3-methylchloride. Then sodium thiomethoxide was added to the reaction mixture to get indole-3-methylthioether (**124**) in 52% yield. Oxidation of indole-3-methylthioether (**124**) with oxone yielded brussalexin B (**117,** Fig **30**, 70% yield).

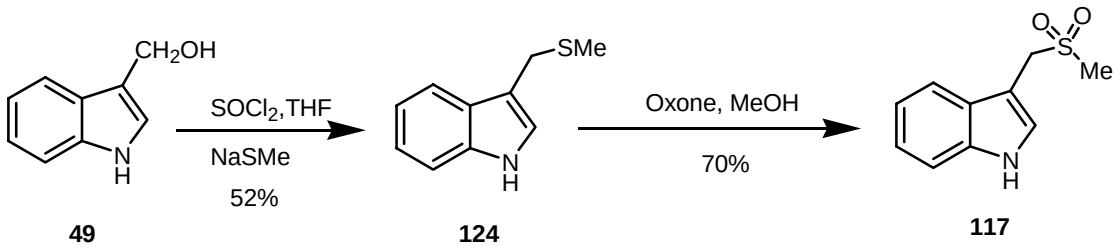


Fig **30**. Synthesis of brussalexin B (**117)**.

Synthesis of brussalexin C (**118**) was accomplished in three steps, via 4-methoxyindole-3-methanol (**126**) and 4-methoxyindole-3-methayl thiomethoxide (**127**) as outlined in Fig. **31**, and the overall yield was 44%. 4-Methoxyindol-3-carboxaldehyde in ethanol was reduced by $NaBH_4$ to yield 4-methoxyindol-3-methanol (**126**). 4-Methoxyindol-3-methanol (**126**) was treated with Et_3N and $SOCl_2$ at 0°C for 40 min to form unstable 4-methoxy indolyl-3-methylchloride. Then sodium thiomethoxide was added to the reaction mixture to get 4-methoxyindole-3-methylthioether (**127**) in 58% yield. Oxidation of 4-methoxy indole-3-methylthioether (**127**) with oxone yielded brussalexin C (**118,** Fig **31**, 60% yield).

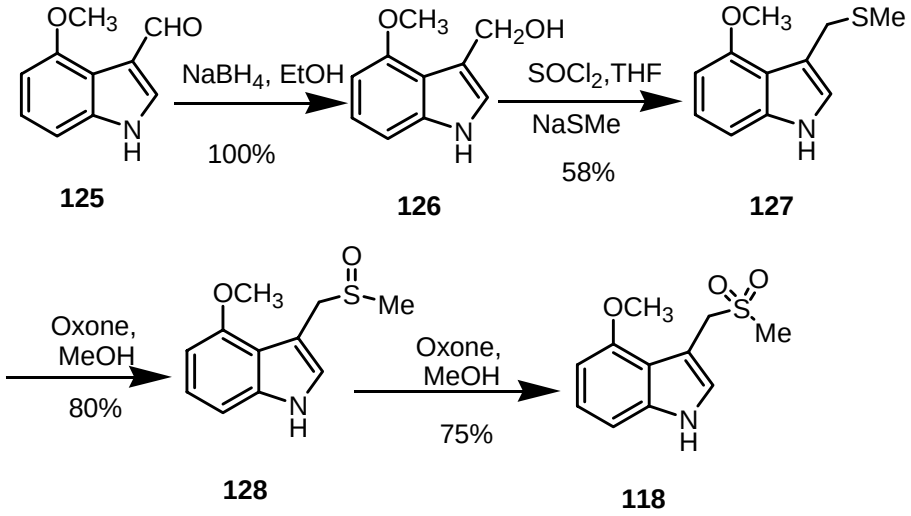


Fig **31**. Synthesis of brussalexin C (**118**).

Synthesis of brussalexin A (**121**) was accomplished in three steps via (indol-3-yl)-methanethiol (**131**) as outlined in Fig **32**. Gramine (**129**) was converted to *S*-3-indolymethylthioacetate (**130**) by reaction with dimethylsulfate and potassium tioacetate (Benghiat & Crooks, 1983). The key intermediate indol-3-yl-methanethiol (**131**) was prepared by hydrolysis of *S*-3-indolylmethylthioacetate (**130**) with degassed KOH solution. Indol-3-yl-methanethiol (**131**) was then reacted with allyisocyanate to form brussalexin A (**121**, 3% overall yield, Fig **32**). Recent work reported the synthesis of Brussalexin A in 55% overall yield (Pedras *et al.*, 2007a).

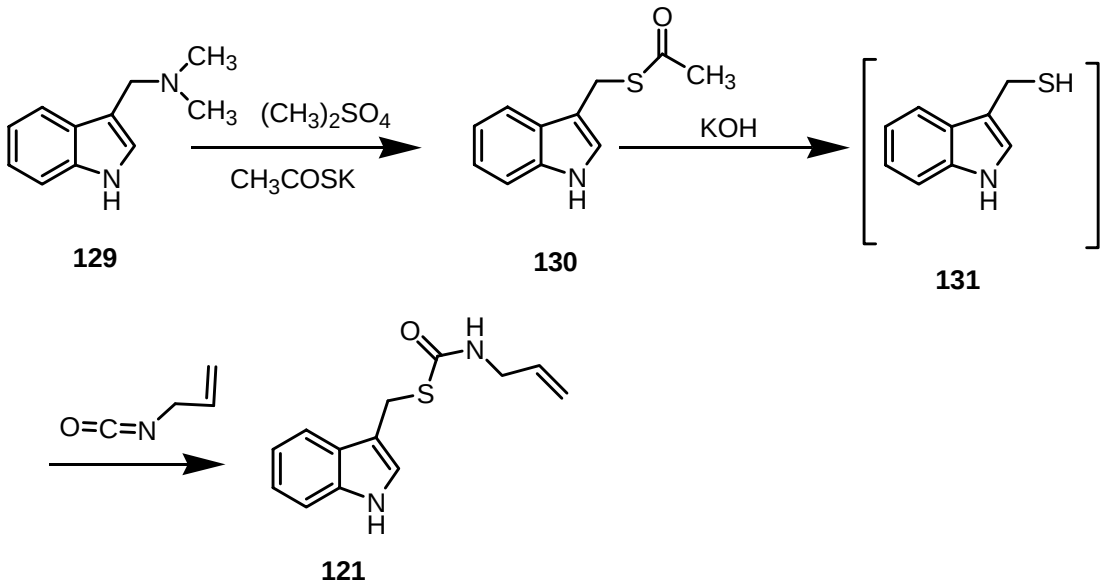


Fig **32**. Synthesis of Brussalexin A (**121**, overall 3 % yield)

2.3.3. Bioassay of metabolites from Brussels sprouts

Antifungal activities of diindolylmethane (**50**), 1-methoxy-3,3'-diindolylmethane (**119**) and brussalexin B (**117**) against fungal pathogens of cruciferous plants (*L. maculans / P. lingam, S. sclerotiorum* and *R. solani)* were established using radial growth antifungal bioassays. Antifungal activity against *C. cucumerinum* was determined using a TLC antifungal bioassay. The antifungal activity of brussalexin B (**117**) was compared with those of structurally related indole-3-methylthioether (**124**). The results of these studies are shown in Table 5. Diindolylmethane (**50**), 1-methoxy-3,3'-diindolylmethane (**119**) appear to exhibit antifungal activity. These compounds diindolylmethane (**50**) and **1**-methoxy-3,3'-diindolylmethane (**119**) cause moderate inhibition of growth of *R. solani, S. sclerotiorum* and *L. maculans* at 1.0×10^{-4} M. Brussalexin B (**117**) and brussalexin C

(**118**) caused little inhibition (5.0×10^{-4} M) of fungal growth on *L. maculans* and *R. solani*, whereas moderate antifungal activity was observed using the same concentration of 3-methylsulfanylmethylindole (**124**). In TLC bioassay 3,3'-diindolylmethane (**50**) caused inhibition of growth *C. cucumerinum* at 1.0×10^{-6} M, but 1-methoxy-3,3'-diindolylmethane (**119**) caused no inhibition of growth at similar concentration.

Table **5**. Antifungal activity [a] (% of inhibition) of diindolylmethane (**50**), 1-methoxy-3,3'-diindolylmethane (**119**), 3-methylsulfanylmethylindole (**124**), brussalexin B (**117**) and brussalexin C (**118**) against pathogens of crucifers: *Leptosphaeria maculans* (*Phoma lingam*) (84 h, incubation), *Rhizoctonia solani* (72 h incubation) and *Sclerotinia sclerotiorum* (48 h incubation).

Metabolites	Conc. (M)	*L. maculans/ P. lingam* [b]	*S. sclerotiorum* [c]	*R. solani* [d]
Diindolylmethane (**50**)	5.0×10^{-4}	71 ± 7	94 ± 4	92 ± 4
	2.5×10^{-4}	62 ± 1	82 ± 4	88 ± 1
	1.0×10^{-4}	32 ± 5	67 ± 1	52 ± 4
	5.0×10^{-5}	8 ± 4	49 ± 2	39 ± 1
1-methoxy-3,3'-diindolylmethane **(**119**)**	5.0×10^{-4}	29 ± 5	-	68 ± 5
	2.5×10^{-4}	6 ± 7	-	56 ± 8
	1.0×10^{-4}	n. i.	49 ± 5	48 ± 1
	5.0×10^{-5}	-	41± 6	24 ± 5
3-Methylsulfanyl methylindole (**124**)	5.0×10^{-4}	59 ± 4	25 ± 1	54 ± 5
	2.5×10^{-4}	21 ± 1	12 ± 1	26 ± 1
	1.0×10^{-4}	9 ± 4	7 ± 3	13 ± 3
Brussalexin B (**117**)	5.0×10^{-4}	22 ± 1	22 ± 2	29 ± 8
	2.5×10^{-4}	14 ± 1	10 ± 4	13 ± 1
Brussalexin C (**118**)	5.0×10^{-4}	23 ± 3	20 ± 2	25 ± 4
	2.5×10^{-4}	10 ± 1	9 ± 2	10 ± 1

[a] % Inhibition = 100 - [(growth on treated/growth on control) × 100] ± standard deviation; results are the means of at least three separate experiments; n.i., no inhibition; n.d., not determined.
[b] Results after 96 hours of incubation.
[c] Results after 48 hours of incubation.
[d] Results after 72 hours of incubation.

2.4. Brown mustard (*Brassica juncea* variety Commercial Brown)

The $CuCl_2$ (1×10^{-2}) elicited brown mustard leaves, were extracted with MeOH. After evaporating the solvent under reduced pressure, the crude extract was dissolved in H_2O and EtOAc. The solvent EtOAc extract was separated and the conc. aqueous extract (49 g) was subjected to reverse phase column chromatography. Fractions F 6, F 7, F 8, and F 10 were further subjected to multiple reverse phase column chromatography to isolate compounds having retention times at R_T = 7.2, 11.8, 14.4, 14.7 and 21.0 min (Albpol, Fig **33**).

The molecular formula of the compound having R_T = 7.2 min (Albpol method) was determined to be $C_9H_{12}NO_2$ by HRMS, which suggested five degrees of unsaturation. The ^{1}H-NMR spectra of this compound indicated it to be phenylalanine (**132**). The NMR data were identical to the authentic sample.

The molecular formula of the elicited compound having R_T = 11.8 min (Albpol method) was measured to be $C_{11}H_{12}N_2O_2$ by HRMS, which suggested seven degrees of unsaturation. The ^{1}H-NMR spectra of this compound indicated it to be tryptophan (**86)**. The HPLC and ^{13}C-NMR data were identical with those of an authentic tryptophan (Fig **34**). The stereochemistry of isolated tryptophan ($[\alpha]_D^{24.5}$ -31; *c* 0.41) was determined to be *S* configuration by comparison of its $[\alpha]_D^{24.5}$ with that of an authentic sample.

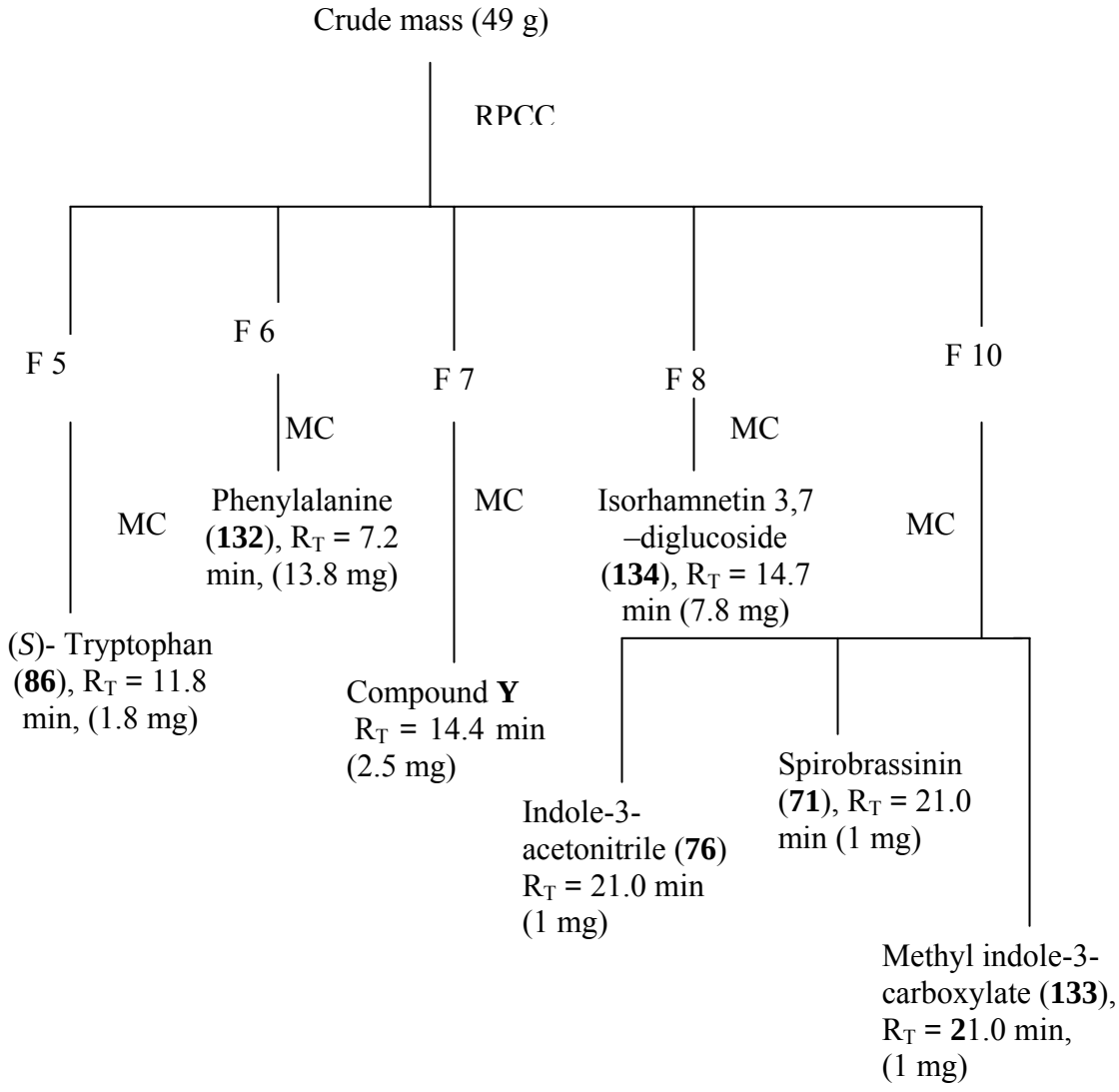


Fig **33**. Isolation of compounds from *Brassica juncea* variety Commercial Brown.

The compound **Y** with R_T = 14.4 min (Albpol method) was isolated through flash column chromatography and reverse phase column chromatography (Fig **33**). High resolution mass spectrometry of the compound **Y** provided the mass of fragments instead of molecular molar mass but LC-MS indicated the molecular ion to be *m/z* 470. The ^{1}H-NMR (in CD_3CN) spectrum of this metabolite indicated four aromatic protons

and eleven other protons in between δ_H 3 to 5 ppm. The UV spectrum and retention time of compound **Y** did not match with any of the previously isolated crucifer metabolites available in Pedras' group (Pedras *et al.,* 2006 and unpublished work in Pedras lab).

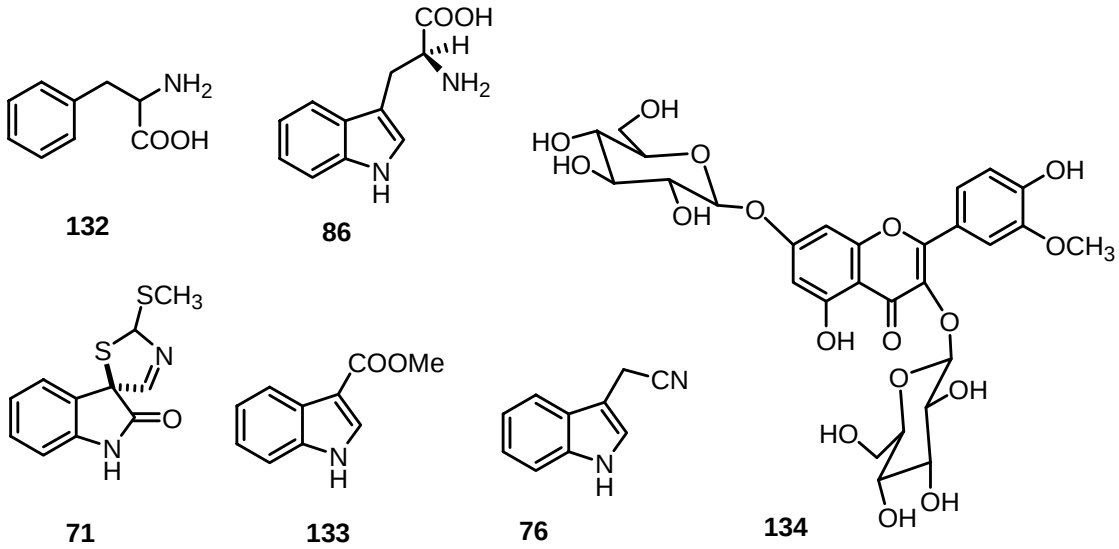


Fig **34**. Phytoalexins spirobrassinin (**71**) and indole-3-acetonitrile (**76**), and compounds phenylalanine (**132**), (*S*)-tryptophan (**86**), isorhamnetin 3, 7-diglucoside **(134),** methyl-idole-3-carboxylate (**133**) were isolated from *Brassica juncea.*

The molecular ion of yellow compound having R_T = 14.7 min (Albpol method) was measured to be *m/z* 640 by HRMS. The UV absorption at 350, 255, and 207 nm indicated it to be a flavonoid compound. The ^{1}H-NMR spectrum of this metabolite showed five aromatic protons at δ_H 7.96, 7.65, 6.92, 6.79 and 6.51 as well as three methoxy protons at δ_H 3.73. Signals at δ_H 5.48 (d, 1H, J = 7 Hz) and δ_H 5.07 (d, 1H, J = 7 Hz) indicated the presence of two β- anomeric protons. LC-MS spectra showed the

loss of two pyranoside units (*m/z* 315, 477, 639 and 640) (Romani *et al.*, 2006). Therefore, the probable structure would be isorhamnetin 3, 7-diglucoside (**134,** Fig **34**). Isorhamnetin 3,7-diglucoside was previously detected in *Brassica juncea* (L) Czern extract (Aguinagald, 1988).

The component with R_T at 21.0 min (Albpol method) revealed to be a mixture of thee compounds. HPLC retention times of these compounds were R_T = 11.8 min (21.0 min in Albpol method) and 12.2 min (Gradscr method). These compounds were purified by flash column chromatography and identified as follows. The compound with R_T = 11.8 min. had the molecular formula $C_{10}H_8N_2$ by HRMS. The HPLC, UV and mass fragmentation (LC-MS) were identical with those of the authentic indole-3-acetonitrile (**76**).

The component with R_T = 12.2 min (21.0 min in Albpol method) was identified as spirobrassinin (71) and it was also isolated from cauliflower. The UV and NMR spectra were identical with that of authentic spirobrassinin (**71**, Pedras *et al.*, 2004d).

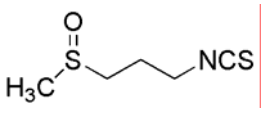
The ^{1}H-NMR (CD_3CN) spectrum of the compound having R_T = 12.2 min indicated the presence of an indole system along with a signal due to a methoxy group at δ_H 3.87 ppm. The compound was assigned as methyl indole-3-carboxylate (**133**). The spectral data were identical to those of an authentic sample.

2.5. Wild species

2.5.1. Metabolites from Asian mustard (*Brassica tournefortii*)

In time course experiments, the extract from the elicited leaves showed a maximum production of induced compounds at 72 hours. HPLC analysis of this extract

showed two new peaks at R_T = 4.5 and 11.8 min. The elicited Asian mustard leaves were harvested and extracted in the same manner that was used in the time course experiment. The extract was subjected to flash column chromatography. Fractions F 8 was further subjected to multiple chromatography to yield a compound with retention time at R_T = 4.5. In FTIR spectrum, a strong absorption at ν 2099 cm^{-1} indicated the presence of isothiocyanide group and absorption at ν 1046 cm^{-1} indicated a sulfoxide group. The ^{1}H-NMR spectrum at δ_H 2.54 (s, 3H) indicated the presence of a thiomethyl group along with three methylene groups at δ_H 3.75 (dd, 2H, *J* = 6.5, 6.5 Hz), 2.82 (m, 1H), 2.71 (m, 1H) and 2.10 (m, 1H). The molecular formula of this compound was determined to be C_5H_9NSO by HRMS, which suggested three degrees of unsaturation. Therefore, the proposed structure 3-(methylsulfinyl) propylisothiocyanate (**135**) was proposed for this metabolite. The optical rotation at $([\alpha]_D^{24.5}$ - 66 c 4.75; CH_2Cl_2 and ^{1}H-NMR spectrum at δ 2.82 (1H, m) and 2.71 (1H, m) indicated the presence of a stereogenic centre. The ^{1}H-NMR and FTIR spectra matched with those of 3-(methylsulfinyl) propylisothiocyanate (**135**) (Holland *et al.,* 1995, Fig **35).** The compound with R_T = 11.8 min. had the molecular formula $C_{10}H_8N_2$ by HRMS. The HPLC, UV and mass fragmentation (LC-MS) were identical with those of the authentic indolyl-3-acetonitrile (**76**).



135

Fig **35**. Compound 3-(methylsulfinyl) propylisothiocyanate (**135**) isolated from Asian mustard.

2.5.2. Metabolites from sand rocket (*Diplotaxis tenufolia*)

In time course experiments, the extract from elicited leaves of sandrocket showed a maximum production of induced compounds at 72 hours. HPLC analysis of this extract showed three peaks at R_T = 11.8, 13.9 and 22.9 min. The elicited leaves were extracted with EtOAc and the residue (1.2 g) was separated by gradient flash column chromatography. Fraction F 4 was further subjected to column chromatography, guided by bioassay against *C. cucumerinum* to isolate elicited compound with HPLC retention time at R_T = 22.9 min. The molecular formula of the elicited compound having R_T = 22.9 min was measured to be $C_{10}H_{16}S_3N_2$ by HRMS, which suggested four degrees of unsaturation. The ^{1}H-NMR spectrum of this compound displaying signals from eight methylene groups and two signals likely due to two CH_2S groups (δ_H 2.44 and 2.76), two CH_2NCS group (δ_H 3.62 and 2.76) and four methylene (δ_H 1.80 and 1.73 at 3:1 ratio). Ten carbons could be distinguished by ^{13}C-NMR including two carbon with δ_C 119.7 ppm indicative of two CH_2NCS group. Based on these data 5-(3-isothiocyanato-propylsulfanyl)-pentylisothiocyanate (**136)** was proposed as the structure of this isolated compund (Fig **36**).

136

Fig **36**. Compound 5-(3-isothiocyanato-propylsulfanyl)-pentylisothiocyanate (**136**) isolated from sand rocket.

2.5.3. Metabolites from wallrocket (*Diplotaxis muralis*)

In time course experiments, the extract from the elicited leaves showed a maximum production of induced compounds at 48 hours. HPLC analysis of this extract showed three new and a constitutive metabolites at R_T = 13.9, 20.1, 24.0 and 28.0 min. In order to isolate these induced metabolites, large scale experiments were carried out. In large scale experiments, plants were elicited by treating with $CuCl_2$ solution. Elicited leaves were collected, frozen in liquid nitrogen, crushed, and extracted with EtOAc. The crude extract of wallrocket (5.7 g) was fractionated by gradient flash column chromatography. Fractions F 4, F 5, F 6, F 7 and F 8 were further subjected to multiple column chromatography and reverse phase column chromatography guided by HPLC analysis to yield the metabolites with retention times R_T = 13.9, 28.0, 20.1, 24.0 and 36.9 respectively (Fig **37**).

The molecular formula of the elicited compound with R_T = 20.1 min was determined to be $C_{12}H_{12}O_2N_2$ by HRMS, which suggested eight degrees of unsaturation. The ^{1}H-NMR spectrum of this metabolite showed four aromatic protons as well as one methylene and six methoxy protons. The spin system of aromatic

protons showed that the proton at δ_H 7.20 (H-6, dd, *J* = 8, 8 Hz) was coupled with two protons at δ_H 7.05 (H-7, d, *J* = 8 Hz) and 6.65 (H-5, d, *J* = 7.5 Hz) suggesting an indole ring having substitution at 4 or 7 position. The signals at δ_H 7.32 (s, H-2) indicated an indole ring with substitution at C-3 position. The signals at δ_H 4.07 (s, 3H), (4.05s, 2H) and 4.00 (s, 3H) indicated one methylene group attached with indole ring at C-3 and two methoxy groups in the indole ring at positions 1 and 4 or 1 and 7. Arvelexin (**84**) and caulilexin C (**105**) are two indolyl-3-acetonitrile having methoxy substitution at 1 or 4 position (Pedras, *et al*., 2004c, Pedras, *et al*., 2006b). Therefore, the structure of this metabolite was assigned as 1, 4-dimethoxyindole-3-acetonitrile (**137**).

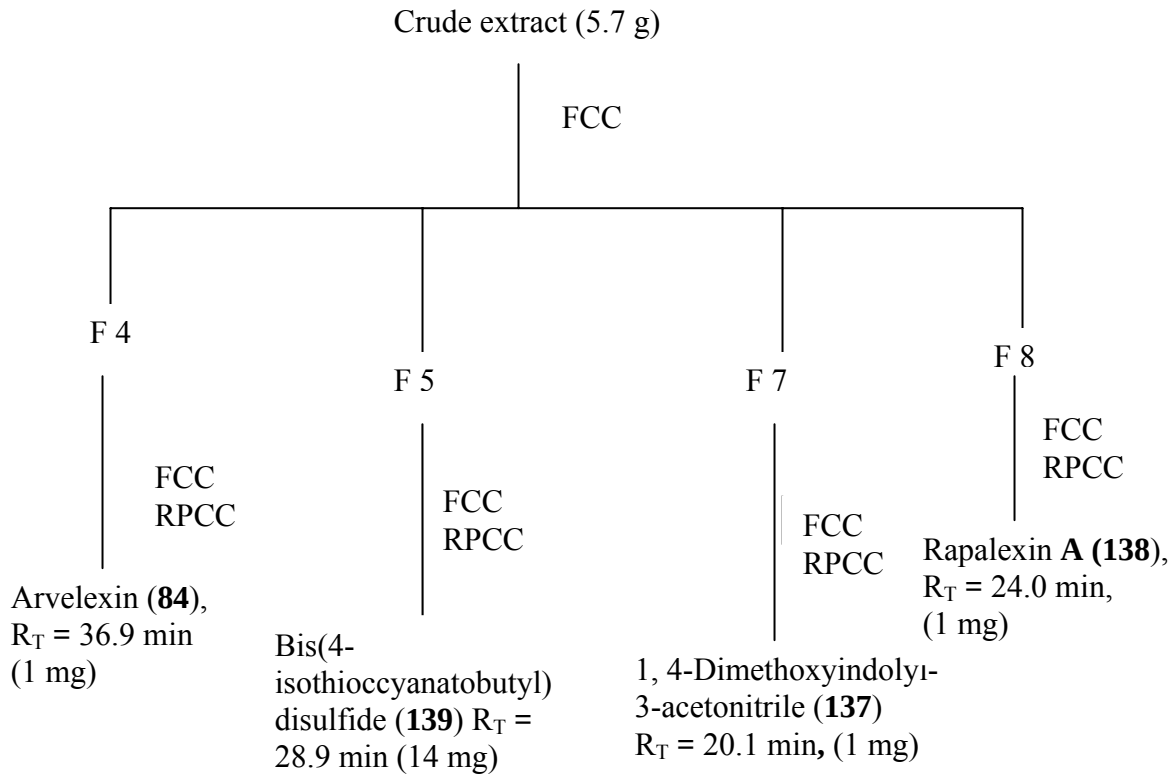
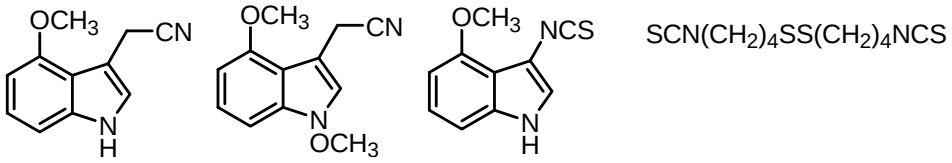


Fig **37**. Isolation of compounds from wallrocket.

The molecular formula of the elicited compound with R_T = 24.0 min was determined to be $C_{10}H_8ON_2$ by HRMS, which suggested eight degrees of unsaturation. The ^{1}H-NMR spectrum of this metabolite showed four aromatic protons, as well as a methoxy group. The spin system of the aromatic protons was similar to that of arvelexin (**84,** Fig **38**). Therefore, this elicited compound with R_T = 24.0 min could have structures **141** or **138**. To confirm the structure, 4-methoxyindole-3-thiocyanate (**141)** was synthesized by coupling 4-methoxyindole (**140**) with ammoniumthiocyanate in presence of iodine. The ^{1}H-NMR of 4-methoxyindole-3-thiocyanate (**141**) did not match with the isolated compound (Fig **39**). The other possible structure is 4-methoxyindole-3-isothiocyanate (**138**). The structure of 4-methoxyindole-3-isothiocyanate (**138**) was recently proven by synthesis (Pedras *et al*., 2007b).

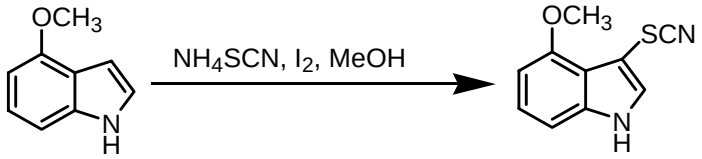


84 **137** **138** **139**

Fig **38**. Compound arvelexin (**84),** 1,4 dimethoxyindole (**137**), rapalexin **A** (**138**) and Bis(4-isothioccyanatobutyl)disulfide (**139**) isolated from wallrocket.

The molecular formula of the metabolites with R_T at 28.0 min was measured to be $C_{10}H_{16}S_4N_2$ by HRMS, which suggested four degrees of unsaturation. Strong doublet absorptions at 2106, 2183 cm^{-1} and 1346 cm^{-1} in the FTIR indicated the presence of an alkyl isothiocyanate group (Svatek *et al.*, 1959). The ^{1}H-NMR spectrum

contained a multiplet at δ_H 1.83 ppm and two triplets at δ_H 2.73 and 3.57 ppm having an integration ratio 2:1:1. The spectral data matched with those of the previously isolated compound bis(4-isothioccyanatobutyl)disulfide (**139**) from *Eruca sativa* (Cerny *et al.*,1996).



140 **141**

Fig **39**. Synthesis of 4-methoxyindole-3-isothiocyanate (**141**).

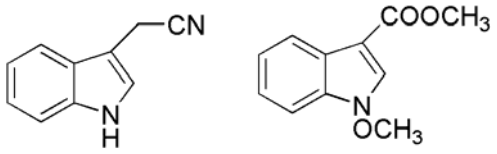
Table **6**. Production[a] of metabolites[b] in wallrocket upon elicitation with $CuCl_2$ (1×10^{-2} M).

Metabolite	24 h	48 h	72 h	96 h	120 h
Arvelexin (**84**)	4.3 ± 1.2	0.2±0.08	0.15±0.09	0.73±0.08	0.026±0.008
Rapalexin A (**138**)	19±6	27±1	200±3	22±2	17±9
1, 4-dimethoxy indolyl-3-acetonitrile (**137**)	-	140	-	-	-
Bis(4-isothio cyanatobutyl) disulfide (**139**)	-	1490	-	-	-

[a]Amount of induced metabolites are 10^{-3}µmol per 100 g of fresh tissue.
[b]Results are presented as means ± standard deviation.

2.5.4. Metabolites from hedge mustard (*Sysimbrium officinale*)

In time course experiments, the extracts from the elicited leaves showed a maximum production of induced compounds at 72 hours. HPLC analysis of this extract showed two new peaks at R_T = 11.8, 13.9 and 19.0 min. The elicited hedge mustard leaves were extracted and the residues were separated by gradient flash column chromatography (C_6H_{12}:CH_2Cl_2:MeOH) as described in the experimental. Fractions F 8 and F 9 were further subjected to multiple chromatographies to yield the elicited compounds with HPLC retention times R_T = 11.8 and 19.0 min. The HPLC and ^{1}H-NMR data of former compound were identical with those of an authentic sample of indolyl-3-acetonitrile (**76**, Pedras *et al*., 2002). The ^{1}H-NMR (CD_3CN) spectrum of compound having R_T = 19.0 min indicated the presence of an indole system along with signals likely due to two methoxy groups at δ_H 3.87 and δ_H 4.22. The ^{1}H-NMR (CD_3CN) spectral data of this metabolite suggested it to be methyl 1-methoxyindole-3-carboxylate (**59,** Fig **40**). The HPLC and ^{1}H-NMR spectra were identical with those of an authentic sample (Pedras and Sorensen, 1998).



76 **59**

Fig **40**. Phytoalexin indole-3-acetonitrile (**76**) and methyl 1-methoxyindole-3-carboxylate (**59**) from hedge mustard.

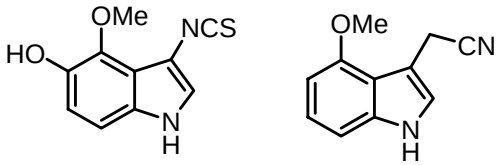
Table **7**. Production[a] of metabolites[b] in hedge mustard upon elicitation with $CuCl_2$ (1×10^{-2} M).

Metabolites	24 h	48 h	72 h	96 h	120 h
Indole-3-acetonitrile **(76)**	0.038±0.007	0.25±0.05	0.64±0.14	0.078±0.019	0.027±0.001
Methyl-1-methoxyindole-3-Carboxylate (**59**)	-	10.5	-	-	-

[a]Amount of induced metabolites are µmol per 100 g of fresh tissue.
[b]Results are presented as mean ± standard deviation.

2.5.5. Metabolites from Abyssinian mustard (*Crambe abyssinica*)

In time course experiments, the extract from elicited Abyssinian mustard leaves showed a maximum production of induced compounds at 72 hours. HPLC analysis of this extract showed four new peaks at R_T = 11.8, 13.9 and 15.0 min. In order to isolate induced metabolites, large scale experiments were carried out. In large scale experiments, plants were elicited by spraying with $CuCl_2$ solution. Elicited leaves were collected, frozen in liquid nitrogen, crushed, extracted with ethyl acetate and the solvent was concentrated. The crude extract was fractionated by gradient flash column chromatography. Fraction F 7 was further subjected to multiple column chromatography and reverse phase column chromatography guided by HPLC analysis to isolate the elicited compounds with retention times R_T = 13.9 and 15.0 min. respectively. The UV, NMR and HRMS data of compound having R_T = 13.9 min were identical with those of arvelexin (**84)** previously isolated from *T. arvense* infected with $CuCl_2$ (Pedras *et al.*, 2004). The HPLC and ^{1}H-NMR data of the compound with R_T = 15 min were identical with those of an authentic sample of rapalexin B (**142**, Fig **41**, Pedras *et al*., 2007b).



142 **84**

Fig **41**. Phytoalexin rapalexin B (**142**)**,** and arvelexin (**84**) from Abyssinian mustard

Table **8**. Production[a] of metabolites[b] in Ayssinian mustard upon elicitation with $CuCl_2$ (1×10^{-2}M).

Metabolites	24 h	48 h	72 h	96 h	120 h
Indole-3-acetonitrile (**76**)	0.29 ± 0.01	1.62 ± 0.67	0.80 ± 0.13	1.19 ± 0.27	0.98 ± 0.06
Arvelexin (**84**)	1.14 ± 0.05	5.1 ± 0.8	1.5 ± 0.25	0.99 ± 0.03	0.62 ± 0.07
Rapalexin B (**142**)	0.0 ± 0.0	11.5 ± 0.6	13.8 ± 2.5	1.20 ± 0.15	5.3 ± 0.38

[a]Amount of induced metabolites are μmol per 100 g of fresh tissue.
[b]Results are presented as mean ± standard deviation.

3. Conclusions

Caulilexin A (**106**), B (**107**) and C (**105**) were the first isolated phytoalexins from cauliflower (Pedras *et al*., 2006b). These are the new phytoalexins in Cruciferae family. Four other known pyhtoalexins, 1-methoxybrassitin (**55**), spirobrassinin (**71**), brassicanal C (**60**) and isalexin (**64**) were also reported from cauliflower. Seven other phytoalexins, cyclobrassinin (**62**), 1-methoxyspirobrassinin (**72**), sinalexin (**83**), arvelexin (**84**), indole-3-acetonitrile (**76**), rutalexin (**69**) and 1-methoxycyclobrassinin (**81**) were detected in the same plant. The first reported synthesis for the cruciferous phytoalexin caulilexin B (**107**) has been carried out. Caulilexin C (**105**) was synthesized in two different pathways (Table **9**, Pedras *et al*., 2006b).

Brussalexin A (**121**), B (**117**) and C (**118**) were the first isolated phytoalexins from Brussels sprouts. These are new phytoalexins from crucifers. One new metabolite 1-methoxy-3,3'-diindolylmethane (**119**) and one known pyhtoalexin, 1-methoxybrassitin along with three other metabolites, ascorbigen (**51**), diindolylmethane (**50**) and di-(1-methoxy-3-indolyl)methane (**120**) were also reported from Brussels sprouts. Two other phytoalexins, arvelexin (**84**) and indole-3-acetonitrile (**76**) were detected in the same plant. The first synthesis of the phytoalexin brussalexin A (**121**), B (**117**) and C (**118**) was carried out (Pedras *et al*., 2007a).

Rapalexin A (**138**), arvelexin (**84**), and phytoanticipin bis-(4-isothiocyanotobutyl) disulfide (**139**) and a new metabolite 1,4-dimethoxyindole-3-acetonitrile (**137**) were isolated from wallrocket (Table **9**).

From four wild species such as hedge mustard, sandrocket, Abyssinian mustard and Asian mustard, number phytoalexins e.g. indolyl-3-acetonitrile (**76**), arvelexin (**84**), methyl-1-methoxyindolecarboxylate (**59**) and rapalexin B (**142**) along with

thio/isothiocyanates e.g. 5-(3-isothiocyanato-propylsulfanyl)-pentylisothiocyanate (**136**) and 3-(methylsulfinyl)propylisothiocyanate (**135**) were isolated (Table **9**).

The cauliflower phytoalexins caulilexin A (**106**) and B (**107**) are the first reported phytoalexins having disulfide and formamide in the structure, respectively. The phytoalexin cauliexin A (**106**) appears to have strong inhibitory effect on *S. scletiorum* and *R. solani* and moderate inhibitory effect on *L. maculans*. The Brussels sprouts phytoalexins brussalexin A (**121**) is the first reported phytoalexin having thiolcarbamate in the structure whereas brussalexin B (**117**) and C (**118**) are also the first reported phytoalexins having methanesulfonyl in the structure. Brussalexin A (**121**), B (**117**) and C (**118**) showed antifungal activity against *L. maculans, S. scletiorum* and *R. solani*. The phytoanticipins diindolylmethane (**50**) and 1-methoxy-3,3'-diindolylmethane (**119**) showed moderate inhibitory effect on *L. maculans, S. scletiorum* and *R. solani.*

Table **9**. Elicited and constitute metabolites isolated or detected in crucifer plants investigated in this project.

Species	Metabolites
Brassica juncea (Brown mustard)	Phenylalanine (**132**, isolated) Tryptophane (**86**, isolated) Indole-3-acetonitrile (**76**, isolated) Spirobrassinin (**71**, isolated) Methylindole-3-carboxylate (**133**, isolated) Isorhamnetin 3,7 -diglucoside (**134**, likely)
Brassica oleracea var. *botrytis* (Cauliflower)	Caulilexin A (**106**, isolated) Caulilexin B (**107**, isolated) Caulilexin C (**105**, isolated)

	1-Methoxybrassitin (**55**, isolated) Spirobrassinin (**71**, isolated) Brassicanal C (**58**, isolated) Isalexine (**64**, isolated) Cyclobrassinin (**62**, detected)[a] Methoxyspirobrassinin (**72**, detected)[a] Sinalexin (**83**, detected)[a] Arvelexin (**84**, detected)[a] Indole-3-acetonitrile (**76**, detected)[a] Rutalexin (**69**, detected)[a] 1-Methoxycyclobrassinin (**81**, detected)[a]
Brassica oleracea var. *gemmifera* (Brussels sprouts)	Brussalexin A (**121**, isolated) Brussalexin B (**117**, isolated) Brussalexin C (**118**, isolated) Diindolylmethane (**50**, isolated) 1-Methoxybrassitin (**55**, isolated) Ascorbigen (**51**, isolated) Indole-3-acetonitrile (**76**, detected)[a] Arvelexin (**84**, detected)[a]
Brassica tournefortii (Asian mustard)	3-(Methylsulfinyl) propylisothiocyanate (**135**, isolated) Indole-3-acetonitrile (**76**, isolated)
C. abyssinica (Abyssinian mustard)	Rapalexin B (**106**, isolated) Arvelexin (**84**, isolated)
Diplotaxis muralis (Wallrocket)	Indole-3-acetonitrile (**76**, detected)[a] Arvelexin (**84**, isolated) 1, 4-Dimethoxyindole-3-acetonitrile (**137**, isolated) Rapalexin **A** (**138**, isolated) Bis(4-isothioccyanotobutyl)- disulfide (**139**, isolated)
Sisybrium officinale (hedge mustard)	Indole-3-acetonitrile (**76**, isolated) Arvelexin (**84**, detected)[a] Methyl-1-methoxyindolecarboxylate (**142**, isolated)

Chapter 4

4. Experimental

4.1. General methods

All chemicals were purchased from Sigma-Aldrich, Canada (Oakville, ON). All solvents were HPLC or ACS grade and used as such except for CH_2Cl_2 and $CHCl_3$, which were redistilled. Thin layer chromatography (TLC) was carried out on pre-coated silica gel aluminium plates (Merck, 60 $F_{254,}$ 20 cm × 20 cm, 0.25 mm thickness). Eluted TLC plates were examined under UV light (254 nm) and were dipped in a 5% (w/v) aqueous phosphomolibdic acid solution containing 1% (w/v) ceric sulfate and 4% (v/v) water, followed by heating on a hot plate. Flash column chromatography (FCC) was performed on silica gel, Merck grade 60, mesh size 230-400, 60 Aº, or on J. T. Baker reversed phase C-18 silica gel, 40 μm.

High performance liquid chromatography (HPLC) analysis was carried out with high performance liquid chromatography systems equipped with quaternary pumps, auto injectors, photodiode array detectors (wave length 190-600 nm), degasser, and hypersil

ODS columns (5 μm particle size silica, 4.6 mm internal diameter × 200 mm) and in-line filters. A gradient elution [CH_3CN:H_2O (25:75) to CH_3CN (100%)] for 35 minutes and flow rate 1.0 ml/min was used. Sample solutions in MeOH, CH_3CN or water were filtered through a tight cotton wool plug before analysis.

Specific Optical rotations ($[\alpha]_D$) were determined using a Digipol 781-TDV auto polarimeter. The solutions were placed in a 1 ml cell, length 10 cm; the concentrations are reported in g/100 ml and the units are 10^{-1} deg cm^2 g^{-1}.

NMR spectra were obtained with Bruker Avance 500 spectrometers (chemical shifts are reported in ppm where δ of TMS is zero). The chemical shifts (δ) values are referred to $CDCl_3$ ($CHCl_3$ at 7.27 ppm), CD_3CN (CD_2HCN at 1.94 ppm) or CD_3OD (CD_2HOD at 3.31 ppm) for ^{1}H-NMR spectra. Assuming first order behaviour, the multiplicities in ^{1}H-NMR are indicated by one or more of the following s = singlet, d = doublet, dd=doublet of doublet, t = triplet, q = quartet, m = multiplet and br = broad. Spin coupling constants (*J*) are reported to the closest 0.5 Hz. For ^{13}C-NMR chemical shifts (δ) values are referred to $CDCl_3$ (77.2 ppm), CD_3CN (118.7 ppm) or CD_3OD (49.2 ppm). 2DNMR experiments performed were COSY-45 (^{1}H-^{1}H correlations), HMQC (^{13}C-^{1}H single bond correlations) and HMBC (^{13}C-^{1}H multiple bond correlations).

Fourier transform infrared (FTIR) spectra were recorded on Bio-Rad FTS-40 spectrometers using the diffuse reflectance method on samples dispersed in KBr.

Mass spectra (MS), high resolution mass spectra (HR-MS), electron impact (EI) or electron spray ionization (ESI) were obtained on a VG 70 SE mass spectrometer.

Gas chromatography – mass spectra (GC-MS) was obtained on a Fisons GC 8000 series model connected to the VG 70 SE mass spectrometer.

4.2. Plant material

Cauliflower, broccoli and Brussels sprouts were purchased in local markets. Seeds of sand rocket (*B. tournefortii*), wallrocket (*D. muralis*), Abyssinian mustard (*C. abyssinica*), brown mustard (*B. juncea* variety Commercial Brown) and hedge mustard (*S. officinale*) were obtained from Plant Gene Resources, AAFC, Saskatoon, Canada. The seeds were sown in a commercial potting soil mixture, and plants were grown in a growth chamber with 16 hours of light (fluorescent and incandescent)/8 hours of dark at 24±2 °C.

4.3. Antifungal bioassays

Bioassays were carried out using the plant pathogens *Rhizoctonia* solani (AG 2-1 isolate), *Leptosphearia maculans* (BJ-125 isolate), *Sclerotinia sclerotiorum* (clone #33) and *Cladosporium cucumerinum. R. solani* and *L. maculans* were grown on potato dextrose agar (PDA) media under continuous light, whereas *S. sclerotiorum* was grown on potato dextrose agar (PDA) media in the dark. Sclerotia were formed in the plates after 15 days and stored at 22 °C. *L. maculans* spores were collected after 15 days and stored at -20 °C. After three days of inoculation, the plates containing *R. solani* were stored.

The antifungal activities of compounds (**50, 58, 60, 84, 105, 106, 107, 117, 118, 119** and **124**) were tested using a mycelial radial growth bioassay. These compounds were

dissolved in DMSO at 5.0×10^{-2} M and serially diluted with PDA media (DMSO concentration in media was 1%) at 50 °C to make overall concentrations of 5.0×10^{-4} M, 2.5×10^{-4} M, 1.0×10^{-4} M, 5.0×10^{-5} M, 2.5×10^{-5} M, 1.0×10^{-5} M, 5.0×10^{-6} M , 2.5×10^{-6} M and 1.0×10^{-6} M. These solutions were poured (2.5 ml) into the wells of sterile six well plates. The control solutions contained only 1% DMSO in PDA media. An agar plug of *L. maculans* or *R. solani* having 5 mm diameter was placed upside down on the middle of each well. After sealing with parafilm, the plates were incubated at 23±1°C under constant light for a few days. The diameter of mycelia were measured at different intervals and compared with those of controls of *R. solan*i (Pedras & Liu, 2004) and *L. maculans* (Pedras & Okanga, 1999). Antifungal activity against *S. sclerotiorum* (Pedras & Ahihonu, 2002) was determined in the same way but using minimal media (having 5% agar), being used instead of the PDA media. Experiments were carried out at least three times.

Bioassay tests against *C. cucumerinum* were conducted on aluminum backed TLC (2 × 20 cm) plates (Pedras & Sorensen, 1998). Samples were applied on plates and the plates were developed in proper solvent systems followed by cool air drying (40 min). *C. cucumerinum* suspension having concentration 1×10^6 spores/ml in double strength PDB media was sprayed on these spotted plates. These plates were then incubated in plastic boxes in a dark humid environment. After 48 hours, the area containing antifungal compounds remained white, whereas the rest of the areas were greenish or gray, due to fungal growth.

4.4. Time course experiments

4.4.1. Hard tissues

Cauliflower and broccoli were cut vertically in 10-15 mm thick slices whereas Brussels sprouts were cut into two pieces. After 24 hours of incubation in covered plastic boxes, the slices were divided into two groups and labelled control and elicited. The slices labeled elicited were irradiated with UV light in a laminar flow hood for 15-20 min on each side. Both the control and elicited slices were then incubated further in plastic boxes at 20 °C. Control and elicited slices were collected at 24 hour intervals for five days. Slices were ground separately in a blender and the ground tissues were separately extracted with ethyl acetate (150 ml). After 12 hours, the macerate tissues were filtered, the filtrates were dried over Na_2SO_4 and concentrated to dryness under reduced pressure. The residues were redissolved in acetonitrile and analysed by HPLC. Known phytoalexins were identified by comparison of their UV spectra and retention times with those of pure metabolites available in Pedras' group (Pedras *et al.*, 2006).

4.4.2. Soft tissues

Plants 3 to 4 weeks old were sprayed with $CuCl_2$ (1×10^{-2} M) and incubated in a growth chamber. Leaves from elicited plants and from control plants were harvested at 24 hour intervals for 5 days. Both control and elicited leaves were separately frozen in liquid nitrogen, crushed and extracted with ethyl acetate (50 ml per replicate). After 24 hours, the crushed leaves were filtered; the filtrates were dried over Na_2SO_4 and concentrated to dryness under reduced pressure. The residues were redissolved in acetonitrile and analysed by HPLC. Known induced compounds were identified by the

comparison of their UV spectra and retention times with those of authentic samples available in pedras groups (Pedras *et al*., 2006 and unpublished work in pedras group).

4.5. Cauliflower (*Brassica oleracea* var. *botrytis*)

4.5.1. Isolation of elicited compounds

Cauliflowers florets (total fresh weight 12 kg) were purchased from local markets and vertically cut in 10-15 mm thick slices/pieces. After 24 hours of incubation at 20 °C in moist covered plastic boxes, slices were irradiated under UV light for 15 min on each side and were incubated further at 20 °C in moist covered plastic boxes. After four days, slices were ground in a blender and the ground tissues were extracted with ethyl acetate. After 12 hours, the mixture was filtered, and the filtrate was concentrated to dryness under reduced pressure (4.05 g). This residue was redissolved in hexane and washed with water. The hexane extract (2.7 g) was chromatographed on silica gel column (15 cm × 5 cm) and eluted with a gradient of CH_2Cl_2:hexane followed by CH_2Cl_2:MeOH. Thirteen fractions were collected and analysed by HPLC: F 6 showed a peak with R_T = 16.8 min, F 8 showed a peak with R_T = 16.2 min, F 10 showed a peak with R_T = 17.8 min and F 11 showed peaks with R_T = 12.2 min, 9.6 min, 8.8 min, 3.8 min along with other compounds. Caulilexin C (**105**, 1 mg) was isolated from F 6 by two successive flash column chromatography (EtOAc:hexane) and reverse phase column chromatography (CH_3CN:H_2O). F 8 was subjected to reverse phase column chromatography (CH_3CN:H_2O), flash column chromatography (EtOAc:hexane) and preparative TLC (5% Et_2O in CH_2Cl_2) to yield caulilexin A (**106**,

1 mg). 1-Methoxybrassitin (**55**, 2 mg) was isolated from F 10 by flash column chromatography (Et_2O:hexane) and reverse phase column chromatography (CH_3CN:H_2O). Spirobrassinin (**71**, 4 mg), caulilexin B (**107**, 1 mg), brassicanal C (**58**, 1 mg) and isalexin (**64**, 1 mg) were isolated from F 11 by flash column chromatography (Et_2O:hexane) and reverse phase column chromatography (CH_3CN:H_2O).

1-Methoxybrassitin **(55)**

HPLC R_T = 17.8 min. UV (HPLC): λ_{max} 225, 280 nm. ^{1}H-NMR(500 MHz,CD_3CN): δ 7.61 (d, *J* = 8 Hz, 1H), 7.45 (d, *J* = 8 Hz, 1H), 7.37 (d, 1H, *J* = 8 Hz), 7.27 (dd, *J* = 7, 8 Hz, 1H), 7.13 (dd, *J* = 7, 8 Hz, 2H), 6.73 (bs, 3H), 4.53 (d, *J* = 5Hz, 1H), 4.07 (s, 3H), 2.30 (s, 3H). HR-MS: m/z 250.0765 (100%, M^+), cal. 250.0776, EI-MS 250.0765 (100%), 219 (46%), 191 (23%), 171 (41%) 128 (29%), 127 (56%), 117 (29%), 74 (61%).

Brassicanal C **(58)**

HPLC R_T = 9.6 min. UV (HPLC): λ_{max} 215, 248, 318 nm. ^{1}H-NMR (500 MHz, CD_3CN) δ_H 10.72 (bs, 1H), 10.34 (s, 1H), 8.24 (d, *J* = 7 Hz, 1H), 7.67 (d, *J* = 8 Hz, 1H), 7.46 (dd, *J* = 7, 8 Hz, 2H), 7.40 (dd, *J* = 7.5, 7 Hz, 1H), 5.40 (bs, 2H), 3.67 (s, 3H), 2.80 (s, 3H).

Isalexin **(64)**

HPLC R_T = 3.8 min. UV (HPLC): λ_{max}210, 238, 350 nm. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.57 (bs, 1H), 7.53 (dd, *J* = 8, 8 Hz, 1H), 7.27 (1H with $CHCl_3$), 6.63 (d, *J* = 8.5 Hz, 1H), 6.47 (d, *J* = 8 Hz, 1H).

Spirobrassinin **(71)**

HPLC R_T = 12.2 min. UV (HPLC): λ_{max}225, 300 nm. Optical rotation: $[\alpha]_D^{24.5}$ -109, (c 0.35, CH_2Cl_2, 85% ee). ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 8.37 (bs, 1H), 7.34 (d, *J* = 7 Hz, 1H), 7.28 (dd, *J* = 8, 8 Hz, 1H), 7.11 (dd, *J* = 8, 8 Hz, 1H), 6.92 (d, *J* = 8Hz, 1H), 4.7 (d, *J* = 15 Hz, 1H), 4.52 (d, *J* = 15 Hz, 1H), 2.64 (s, 3H).

Caulilexin A (**106**)

HPLC R_T = 16.2 min. ^{1}H-NMR (500 MHz, CD_3CN): δ_H 10.42 (br s, D_2O exchangeable, 1H), 10.20 (s, 1H), 8.09 (d, *J* = 8 Hz, 1H), 7.56 (d, *J* = 8 Hz, 1H), 7.33 (dd, *J* = 8, 8 Hz, 1H), 7.28 (dd, *J* = 8, 8 Hz, 1H), 2.60 (s, 3H). ^{13}C-NMR (125 MHz, CD_3CN): δ 185.5 (s), 143.8 (s), 138.1 (s), 128.0 (s), 126.7 (s), 125.4 (d), 124.1 (d), 121.0 (d), 113.1 (d), 24.1 (q). HREIMS m/z [M+] measured: 223.0121 (223.0126 calc. for $C_{10}H_9NOS_2$); EIMS m/z (% relative abundance): 223 [M+] (58), 176 (100), 121 (19), 77 (16). FTIR ν max: 3213, 2920, 1632, 1578, 1433, 1373, 1242, 846, 745 cm^{-1}.

UV (MeOH) λ max (log ε): 213 (4.4), 252 (4.2), 307 (4.0) nm.

4.5.2. Syntheses of elicited compounds

4.5.2.1. Synthesis of caulilexin C (105)

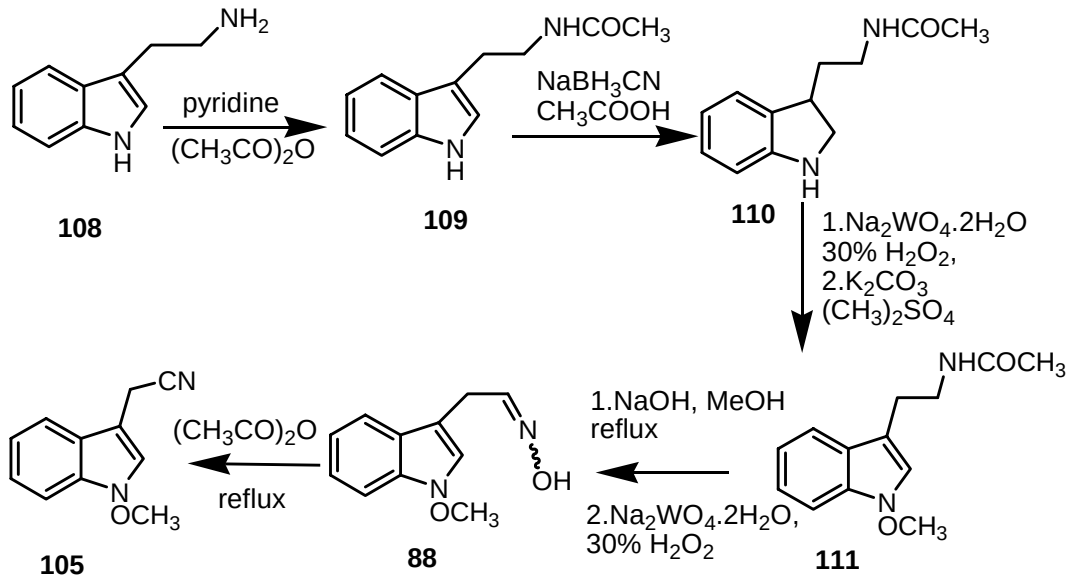


Fig **23**. Synthesis of caulilexin C (**105,** overall yield 10%).

N_b -Acetyltryptamine **(109)**

Tryptamine (**108,** 100 mg, 0.62 mmol), acetic anhydride (400 µl), and pyridine (200 µl) were stirred for 30 min at room temperature. The reaction mixture was diluted with CH_2Cl_2 (1 ml), washed with saturated $NaHCO_3$ solution and then the solvent was evaporated under reduced pressure to yield crude N_b-acetyltryptamine (**109,** 114 mg, yield 90%). Spectral data of N_b-acetyltryptamine (**109**) were identical with those of an authentic sample (Fig **23,** Pedras *et al*., 2004f).

^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 8.27 (s, 1H), 7.62 (d, *J* = 8 Hz, 1H),7.40 (d, *J* = 8 Hz, 1H), 7.23 (dd, *J* = 8, 8 Hz 1H), 7.15 (dd, *J* = 8, 8 Hz 1H), 7.04 (s, 1H), 5.58 (bs, 1H), 3.62 (dd, *J* = 6.5, 6.5 Hz 2H), 3.00 (dd, *J* = 6.5, 6.5 Hz 2H), 1.94 (s, 3H).

2, 3-Dihydro-N_b-acetyltryptamine **(110)**

N_b-Acetyltryptamine (**109,** 110 mg, 0.54 mmol) was dissolved in glacial acetic acid (2 ml) and stirred for 5 minutes. To this solution, $Na(CN)BH_3$ (68 mg, 1.6 mmol) was added in three portions while stirring was allowed to continue. After 3 hours, the reaction mixture was diluted with water (10 ml), made alkaline with 2N NaOH and extracted with CH_2Cl_2 (30 ml × 3). The combined extract was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to yield crude 2,3-dihydro-N_b-acetyltryptamine (**110**, 93 mg, 0.46 mmol, yield 85%). Spectral data of 2,3-dihydro-N_b-acetyltryptamine (**110**) were identical with those of an authentic sample (Fig **23**, Taniguchi & Hino, 1981).

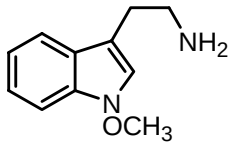
^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.10 (d, *J* = 7.5 Hz, 1H), 7.05 (dd, *J* = 7.5, 7.5 Hz, 1H), 6.74 (dd, *J* = 7.5, 7.5 Hz, 1H), 6.67 (d, *J* = 7.5 Hz, 2H), 5.89 (br s, D_2O exch., 1H), 3.71 (dd, *J* = 8.5,8.5 Hz,1H), 3.31 (m, 2H), 3.24 (m, 2H), 3.12 (br s, D_2O exch, 1H), 1.99 (s, 3H).

***1-Methoxy-N_b-acetyltryptamine* (111)**

A solution of 2,3-dihydro-N_b-acetyltryptamine (**110**, 105 mg, 0.51 mmol) in methanol (3 ml) was treated with a solution of $Na_2WO_4.2H_2O$ (43 mg, 0.13 mmol) in H_2O (260 µl). The suspension was cooled to -15 °C and 30% H_2O_2 solution (600 µl 30% H_2O_2 and 600 µl MeOH) was added to the mixture over 6 min. After 10 min, K_2CO_3 (600 mg, 4.43 mmol) and $(CH_3)_2SO_4$ (160 µl) were added to the mixture with vigorous stirrings at room temperature for 3 hours. The reaction mixture was diluted with water (5 ml) and extracted with Et_2O (30 ml × 3). The combined diethylether extract was dried over Na_2SO_4 and concentrated. The 1-methoxy-N_b-acetyltryptamine (**111**) was purified with flash column chromatography (CH_2Cl_2-MeOH, 98:2) to yield 53 mg (0.28 mmol, 55 %, Fig **23**). Spectral data of 1-methoxy-N_b-acetyltryptamine (**111**) were identical with those of an authentic sample (Iwaki, *et al.*, 2005).

^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.59 (d, *J* = 8 Hz, 1H), 7.44 (d, *J* = 7.5 Hz,1H), 7.28 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.14 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.13 (s,1H), 5.5 (br s, D_2O exch, 1H), 4.09 (s, 3H), 3.60 (dd, *J* = 6.4, 6.4 Hz, 1H), 2.95 (dd, *J* = 6.5, 6.5 Hz, 1H), 1.97 (s, 3H).

1-Methoxytryptamine



1-Methoxy-N_b-acetyltryptamine (**111**, 350 mg, 1.51 mmol) in 15% NaOH (2.1 g NaOH in 15 ml of MeOH) was heated at 124 °C for 12 hours. The solvent was

evaporated and the residue was dissolved in water (10 ml) and extracted with CH_2Cl_2 (50 ml × 3). The combined organic extract was dried over Na_2SO_4 and concentrated. Separation by flash column chromatography (CH_2Cl_2-MeOH-NH_4OH, 98:2-90:10) yielded 1-methoxytryptamine (145 mg, 0.75 mmol, 50 % yield). Spectral data of 1-methoxytryptamine were identical with those of an authentic sample (Somei *et al.*, 1985).

^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.58 (d, J=7.5 Hz, 1H), 7.39 (d, *J* = 8 Hz, 1H), 7.21 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.20(d, *J* = 8 Hz, 1H), 7.073 (dd, *J* = 7.5, 7.5 Hz, 1H), 4.25 (br s, D_2O exch, 2H), 4.03 (s, 3H), 3.16 (bs, 2H), 3.10 (s, 2H).

1-Methoxyindole-3-acetaldoxime **(88)**

To a solution of 1-methoxytryptamine (217 mg, 1.10 mmol) in MeOH (1.5 ml) cooled to -17 °C, $Na_2WO_4.2H_2O$ (6.3 mg, 1.9×10^{-2} mmol in 250 μl H_2O) and 30% H_2O_2 (250 μl) were added dropwise over 4 min. After stirring for 40 min, the mixture was diluted with water (5 ml) and extracted with CH_2Cl_2 (10 ml × 3). The combined organic extract was dried over Na_2SO_4 and concentrated. After separation by flash column chromatography (CH_2Cl_2-MeOH, 98:2), 1-methoxyindole-3-acetaldoxime (**88**) was obtained in 78 mg (0.45 mmol) in 41% yield based on recovery of starting material (Fig **23**). Spectral data of 1-methoxyindole-3-acetaldoxime (**88**) were identical with those of an authentic sample (Pedras & Montaut, 2004f).

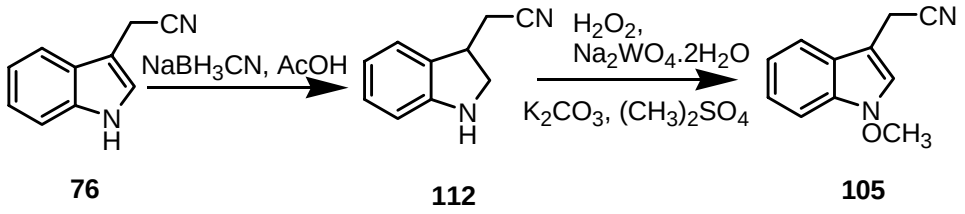
^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.60 (d, 1H, *J* = 7.5 Hz), 7.42 (s 1H), 7.25-7.22 (m, 2H), 7.14 (d, 2H, *J* = 7.5 Hz), 6.7(s, 1H), 4.08 (s, 3H), 3.77 (d, 1H, *J* = 5.5 Hz), 3.68 (d, 1H, *J* = 5.5 Hz).

Synthesis of caulilexin C **(105)**

1-Methoxyindole-3-acetaldoxime (**88**, 95 mg, 0.46 mmol) in acetic anhydride (1 ml) was reflux at 124 °C for 60 min. The reaction mixture was cooled and the solvent was evaporated to dryness. Separation by flash column chromatography (CH_2Cl_2-MeOH, 98:2) gave caulilexin C (**105**, 36 mg, 0.29 mmol) in 64% yield (based on recovered of starting material) (Fig **23**, Pedras *et al*., 2006b).

HPLC R_T = 16.8 min. ^{1}H-NMR (500 MHz, CD_3CN): δ 7.65 (d, *J* = 8 Hz, 1H), 7.50 (d, *J* = 8 Hz, 1H), 7.49(s, 1H), 7.31(dd, *J* = 8, 8 Hz, 1H), 7.31(dd, *J* = 7, 8 Hz, 1H), 7.19 (dd, *J* = 8, 8 Hz, 1H), 4.10 (s, 3H), 3.91 (s, 1H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 133.4 (s), 142.1 (s), 136.2 (d), 127.3 (s), 124.4 (d), 123.3 (d), 120.3 (s), 117.7 (s), 111.2, 14.5. HREIMS *m/z* (% relative abundance) measured: 186.0795 (186.0793 calc. for $C_{11}H_{10}N_2O_2$); EIMS *m/z* (% relative abundance): 186 [M^+] (97), 171 (37), 155 (100), 146 (28), 128 (58), 101 (23), 77 (16). FTIR ν_{max}: 3398, 3124, 3056, 2936, 2249, 1729, 1704, 1453, 1414, 1358, 1240, 1227, 1150, 954, 738 cm^{-1}. UV (CH_2Cl_2) λ_{max} (log ε): 200 (4.4), 219 (4.5), 271 (3.8).

***Synthesis of 1-methoxyindolyl-3-acetonitrile (105) from indolyl-3-acetonitrile* (76).**



76 **112** **105**

Fig **24**. Synthesis of caulilexin C (**105,** overall yield 10%).

To a stirred solution of indolyl-3-acetonitrile (**76**, 100 mg, 0.64 mmol) in glacial acetic acid (2 ml), sodium cyanoborohydride (120 mg, 1.8 mmol) was added in portions over 3 hours. The reaction mixture was diluted with water (40 ml) and extracted with CH_2Cl_2 (50 ml × 3); the combined extract was dried over Na_2SO_4 and concentrated. The crude reaction mixture was fractionated by flash column chromatography to yield 2, 3-dihydroindole-3-acetonitrile (**112**, 40 mg, 60%, Fig **24**).

A vigorously stirred solution of 2,3-dihydroindolyl-3-acetonitrile (**112**, 40 mg) in methanol (2 ml) was treated with a solution of $Na_2WO_4.2H_2O$ (22 mg, 2.2 mmol) in H_2O (160 µl). The resultant suspension was cooled to -18°C and 30% H_2O_2 (300 µl) was added over 15 min. After 30 min, K_2CO_3 (300 mg) and $(CH_3)_2SO_4$ (80 µl) were added to the reaction mixture and the mixture was stirred at room temperature for five hours, the reaction mixture was diluted with water (5 ml), the mixture was extracted with CH_2Cl_2 (10 ml × 3). The combined extract was dried over Na_2SO_4 and concentrated. The extracted was fractionated by flash column chromatography

(EtOAc:hexane, 3:7) to yield 1-methoxyindolyl-3-acetonitrile (**105**, 7 mg, 14% yield, Fig **24**, Pedras *et al*., 2006b).

4.5.2.2. Synthesis of caulilexin B (**107**)

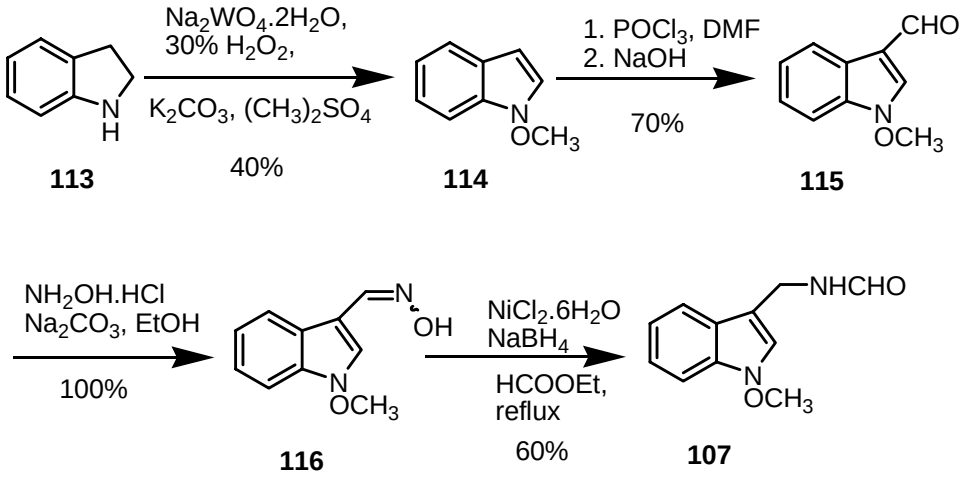


Fig **25**. Synthesis of caulilexin B (**107**)

1-Methoxyindole **(114)**

A solution of indoline (**113**, 556 µl, 4.6 mmol) in methanol (20 ml) was treated with a solution of $Na_2WO_4.2H_2O$ (294 mg, 0.89 mmol) in H_2O (2 ml). The resultant suspension was cooled to -15 °C and 30% H_2O_2 solution (4.5 ml 30% H_2O_2 and 4.5 ml MeOH) was added to the mixture over 15 min while stirring. After 30 min, K_2CO_3 (2.8 g, 20 mmol) and $(CH_3)_2SO_4$ (0.75 ml, 7.5 mmol) were added to the mixture with vigorous stirring at room temperature. After 1.5 hours, the reaction mixture was diluted with water (20 ml) and extracted with Et_2O (50 ml × 3). The combined diethylether

extract was dried over Na_2SO_4 and concentrated. Crude 1-methoxyindole (**114**) was purified with flash column chromatography (CH_2Cl_2-MeOH, 98:2) to yield 352 mg (2.4 mmol, 48 %, Fig **25**). Spectral data of 1-methoxyindole (**114**) were identical with those of an authentic sample (Somei, *et al*., 1985, Kawasaki & Somei, 1990).

^{1}H-NMR (500 MHz, CD_3CN): δ_H 8.23 (s, 1H) 7.15 (t, J = 8, 8 Hz, 1H), 7.12 (t, J = 3, 2.5 Hz, 1H), 7.04 (d, J = 8 Hz, 1H), 6.69 (bs, 1H), 6.49 (d, J = 7.5 Hz, 1H), 3.96 (s, 3H).

1-Methoxyindole-3-carboxaldehyde **(115)**

To a solution of 1-methoxyindole (**114**, 130 mg, 0.88 mmol) in dry DMF (600 µl), phosphorus oxychloride (84 µl, 0.91 mmol) was added slowly at room temperature. After 30 min, the reaction mixture was neutralized with 5N NaOH (2 ml) and refluxed for 5 min. The reaction mixture was extracted with Et_2O (3 ml × 3); the combined organic phase was dried over Na_2SO_4 and concentrated. The product was separated by flash column chromatography (CH_2Cl_2:MeOH, 98:2) to yield 1-methoxyindole-3-carboxaldehyde (**115**, 72 mg, 58 % yield, Fig **23**). Spectral data of 1-methoxyindole-3-carboxaldehyde (**115**) were identical with those of an authentic sample (Pedras & Okinyo, 2006a).

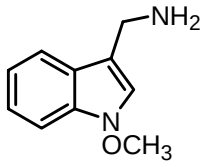
HPLC R_T =12.2 min. ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 10.01 (s, 1H), 8.33 (d, J = 8 Hz, 1H), 7.91(s, 1H), 7.50 (d, J = 8 Hz, 1H), 7.40(dd, J = 8, 8 Hz, 1H), 7.37 (dd, J = 8, 8 Hz, 1H), 4.21(s, 3H).

***1-Methoxyindole-3-carboxaldehyde oxime* (116)**

An aqueous solution of $NH_2OH{\cdot}HCl$ (67 mg, 0.94 mmol) and Na_2CO_3 (51 mg, 0.48 mmol) was added to a solution of 1-methoxyindole-3-carboxaldehyde (**115**, 85 mg, 0.54 mmol). The reaction mixture was heated at 60º C for 3 hours, then the reaction mixture was diluted with water (10 ml) and extracted with Et_2O (20 ml × 3). The combined organic phase was dried over anhydrous Na_2SO_4 and concentrated to dryness to yield 1-methoxyindole-3-carboxaldehyde oxime (**116**, 86 mg, 0.85 mmol, 90% yield). Spectral data of 1-methoxyindole-3-carboxaldehyde oxime (**116**) were identical with those of an authentic sample (Pedras & Okinyo, 2006a).

HPLC R_T =12.3 and 12.5 min (ratio 1:2 for *E* and *Z* isomer)

1-Methoxyindolyl-3-methanamine



To a stirred cooled solution of 1-methoxyindole-3-carboxaldehyde oxime (**116**, 34 mg, 0.19 mmol) in MeOH (500 μl) cooled 0 º C, $NaBH_3(CN)$ (113 mg, 1.79 mmol) and NH_4OAc (50 mg, 0.65 mmol) were added and then a neutralized solution of $TiCl_3$ (30% wt in 2N HCl, 730 μl) was added. After 15 min, the reaction mixture was diluted with water (10 ml), neutralized with 5N NaOH and extracted with CH_2Cl_2 (10 ml × 3). The combined organic phase was dried over anhydrous Na_2SO_4 and concentrated to dryness to yield crude amine (50 mg). After flash column chromatography with

(CH_2Cl_2:MeOH, 9:1), 1-methoxyindole-3-methylamine was obtained in 81 % yield (26 mg). Spectral data of 1-methoxyindole-3-methylamine were identical with those of an authentic sample (Pedras *et al.*, 2006b).

^{1}H-NMR (500 MHz, CD_3OD): δ_H 7.58 (d, J = 8 Hz, 1H), 7.36 (d, J = 8 Hz, 1H), 7.33 (s, 1H), 7.18 (dd, J = 8, 7.5 Hz, 1H), 7.04 (dd, J = 7.5, 7.5 Hz, 1H), 4.02 (s, 3H), 3.92 (d, J = 2 Hz, 1H).

Caulilexin B **(107)**

1-Methoxyindolyl-3-methanamine (34 mg, 0.2 mmol) was refluxed in HCOOEt (2 ml) at 70 ºC for 16 hours. The reaction mixture was allowed to cool to room temperature and the excess HCOOEt was evaporated. Separation by flash column chromatography (CH_2Cl_2: MeOH, 95:5) gave caulilexin B (**107**, 23 mg, 85% yield, Fig **25**) (Pedras et al., 2006b).

HPLC R_T = 8.8 min; ^{1}H-NMR (500 MHz, CD_3CN) δ_H 8.15 (s, 1H), 7.63 (d, 1H, J = 8 Hz), 7.46 (d, 1H, J = 8 Hz), 7.38(s, 1H), 7.27 (dd, 1H, J = 7.5, 7.5 Hz), 6.70 (bs, 1H), 4.52 (d, 2H, J = 6 Hz), 4.07 (s, 3H). ^{13}C-NMR (125 MHz, CD3CN): δ_C 162.2 (s), 133.8 (s), 124.3 (s), 124.0 (d), 123.8 (d), 121.2 (d), 120.6 (d), 110.6 (s), 109.7 (d), 67.0 (q), 33.7 (t); HR-EIMS: m/z (% relative abundance) measured: 204.0898 (92%, 204.0898 calc for $C_{11}H_{12}N_2O_2$); EIMS *m/z* (% relative abundance): 204 (M^+), 175 (22), 173 (64), 146 (21) 145 (39), 118.0659 (100), 117 (31), 91 (21). FTIR ν_{max}: 3282, 3053, 2935, 2860, 1661, 1523, 1452, 1360, 1226, 1101, 740 cm^{-1}. UV (MeOH) λ_{max} (log ε): 271 (4.4), 290 (3.7).

4.6. Brussels sprouts (*Brassica oleracea* var. *gemmifera*)

4.6.1. Isolation of metabolites

Brussels sprouts were cut into two pieces. After 24 hours of incubation at 20 °C in moist covered plastic boxes, the elicited slices were irradiated with UV light for 20 min, incubated in moist covered plastic boxes and after 72 hours were ground in a blender. The ground tissues were stirred in ethyl acetate (3.9 Kg in 6 l). After 12 hours, the mixture was filtered; the filtrate was dried over Na_2SO_4 and concentrated to dryness under reduced pressure (crude extract 9.6 g). This extract was redissolved in hexane and H_2O. The hexane layer was separated, dried over Na_2SO4 and concentrated to dryness. The crude hexane extract (6.7 g) was separated by flash column chromatographed using a gradient elution (CH_2Cl_2:hexane, CH_2Cl_2:MeOH). Thirteen fractions were collected and analysed by HPLC: F 6 showed a peak at R_T 28.4 min, F 7 showed a peak at R_T 23.1 min, F 8 showed peaks at R_T = 33.3 and 36.0 min, F 11 showed a peak at R_T = = 7.2 min, F12 showed a peak at R_T = 8.7 and F 13 showed peaks at R_T = 18.0 and 4.5 min along with other compounds. 1-Methoxy-3,3'-diindolylmethane (**119**) was isolated from F 6 by reverse phase column chromatography (CH_3CN:H_2O). Diindolylmethane (**50**) was isolated from F 7 by reverse phase column chromatography (CH_3CN:H_2O). Di-(1-methoxy-3-indolyl)methane (**120**) was isolated from F 9 by PTLC (CH_2Cl_2:hexane) and reverse phase column chromatography (CH_3CN: H_2O). Brussalexin B (**117**) was isolated from F 11 by flash column chromatography (Et_2O:hexane) and reverse phase column

chromatography (CH_3CN:H_2O). 1-Methoxybrassitin (**55**) and brussalexin C (**118**) was isolated from F 12 by flash column chromatography (Et_2O:hexane) and reverse phase column chromatography (CH_3CN:H_2O). Brussalexin A (**121**) and ascorbigen (**51**) were isolated from F 13 by preparative TLC (CH_2Cl_2:hexane) and reverse phase column chromatography (CH_3CN:H_2O).

Compound **X**

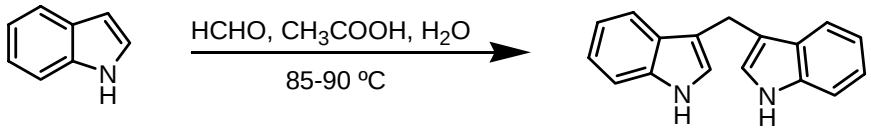
HPLC: R_T = 36.0 min. (Gradscr), 16.4(nonpolarscr). ^{1}H-NMR (500 MHz, CD_3CN) δ_H 9.81(bs, 1H exchangeable with D_2O), 9.06 (bs, 2H exchangeable with D_2O), 8.81 (s, 1H), 8.18 (d, *J* = 7.5 Hz, 2H), 7.77 (d, *J* = 2.4 Hz, 1H), 7.67 (d, *J* = 8 Hz, 1H), 7.41 (d, *J* = 8 Hz, 2H), 7.36 (m, 3H), 7.28 (dd, *J* = 7, 7.5 Hz, 1H), 7.20 (dd, *J* = 7,7.5 Hz, 2H), 7.13 (dd, *J* = 8,7 Hz, 1H). ^{13}C-NMR (125 MHz, CD_3CN): δ_C 150.3, 140.8, 139.3, 137.2, 126.9, 125.3, 125.1, 124.3, 122.5, 120.1, 119.9, 119.8, 119.2, 112.4, 110.8, 110.1, 108.7, 99.7. UV (HPLC): λ_{max} 215, 278, 310, 361 nm.

Ascorbigen **(51)**

HPLC R_T = 4.5 min. UV (HPLC): λ_{max} 222, 280 nm. ^{1}H-NMR (500 MHz, CD_3CN): δ_H 9.24 (bs, 1H D_2O exchangeable), 7.58 (d, *J* = 8Hz, 1H), 7.41 (d, *J* = 8Hz, 1H), 7.21 (s, 1H), 7.13 (dd, *J* = 7.5,7.5 Hz, 1H), 7.04 (dd, *J* = 7.5,7 Hz, 1H), 4.30 (bs, 1H), 4.06 (m, 1H), 4.00 (s, 2H), 3.39 (m, 1H), 3.21 (d, *J* = 14 Hz, 1H). ^{13}C-NMR (125 MHz, CD_3CN): δ_C 175.2, 135.3, 122.2, 124.8, 120.7, 118.3, 118.1, 110.6, 106.7, 106.1, 85.6, 78.4, 74.1, 73.2, 29.3. HREIMS m/z measured: 305.0989 (305.0899 calc. for $C_{15}H_{15}NO_6$.

4.6.2. Synthesis of metabolites

4.6.2.1. Synthesis of diindolylmethane (50)



122 **50**

Fig **28**. Synthesis of diindolylmethane (**50**).

Indole (**122**, 100 mg, 0.85 mmol) was added to a solution of acetic acid (40 µl, 0.68 mmol in 1 ml water). After being stirred for 10 min, 37% aqueous formaldehyde (10 µl) was added to the reaction mixture and heated at 85-90 °C for 5 hours. The mixture was cooled at room temperature, filtered and concentrated. The crude reaction mixture was subjected to reverse phase column chromatography (CH_3CN:H_2O, 75:25) to afford diindolylmethane (**50**, 100 mg, 94% yield, Fig **28**, Jackson *et al*., 1987).

HPLC: R_T = 23.1 min. ^{1}H-NMR (500 MHz, CD_3CN): δ_H 9.03 (s, 2H), 7.54 (d, *J* = 8 Hz, 2H), 7.38 (d, *J* = 8 Hz, 2H), 7.11 (dd, *J* = 7, 7 Hz, 2H), 7.07 (d, *J* = 2 Hz, 2H), 6.99 (dd, *J* = 7, 8 Hz, 2H), 4.22 (s, 2H). HREIMS *m/z* (% relative abundance) measured: 246.1153 (246.1157 calc. for $C_{17}H_{14}N_2$). FTIR ν_{max}: 3413, 3053, 2936, 1618, 1486, 1455, 1421, 1338, 1220, 1089, 1009, 741 cm^{-1}. UV (CH_2Cl_2) λ_{max} (log ε): 224 (4.8), 270 (4.1).

4.6.2.2. Synthesis of 1-methoxy-3, 3'-diindolylmethane (119)

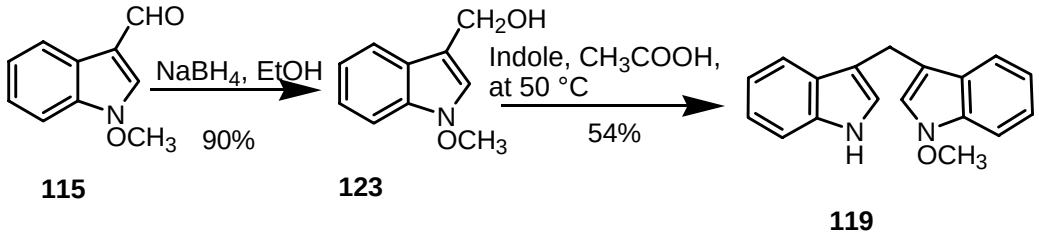


Fig **42**. Synthesis of 1-methoxy-3,3'-diindolylmethane (**119**).

1-Methoxyindole-3-methanol **(123)**

To a stirred solution of 1-methoxyindole-3-carboxaldehyde (**115**, 18 mg, 0.10 mmol) in EtOH (300 µl), $NaBH_4$ (5.2 mg, 0.14 mmol) was added at 0°C; after 1.5 hour, the reaction mixture was diluted with water (5 ml) and extracted with EtOAc (5 ml × 3). The combined organic portion was dried over anhydrous Na_2SO_4 and concentrated to give 1-methoxyindole-3-methanol (**123,** 20 mg, 98% yield, adapted from Letxague *et al*., 1991).

^{1}H-NMR (500 MHz, CD_3CN): δ_H 7.82(d, J = 7.5 Hz, 1H), 7.28 (s, 1H), 7.25 (dd, J = 8, 8 Hz, 1H), 6.73 (d, J = 7.5 Hz, 1H), 4.75 (s, 3H), 3.96 (s, 2H). UV (CH_2Cl_2) λ_{max} (log ε): 220 (4.4), 271 (3.7).

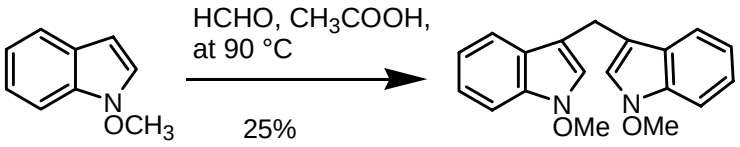
1-Methoxy-3,3-diindolylmethane **(119)**

To a solution of 1-methoxyindole-3-methanol (**123**, 20 mg, 0.098 mmol) and indole (**122**, 24 mg, 0.205 mmol) in EtOH (300 µl), glacial acetic acid (13 µl, 0.22 mmol) was added at 50°C and stirred for 5 hours. The solvent was evaporated to

dryness. The crude reaction mixture was subjected to reverse phase column chromatography (CH_3CN:H_2O-50:50) to afford 1-methoxy-3,3-ddiindolylmethane (**119**, 15 mg, 54%, Fig **42**, adapted from Jackson *et al*., 1987).

HPLC: R_T = 29.3 min. ^{1}H-NMR (500 MHz, CD_3CN) δ_H 9.06 (bs, 1H), 7.56 (d, 1H, *J* = 8 Hz), 7.55 (d, 1H, *J* = 8 Hz), 7.40 (dd, 2H, *J* = 8, 8 Hz), 7.22 (s, 1H), 7.21 (d, 1H, *J* = 8 Hz), 7.13 (d, 1H, *J* = 7.5 Hz), 7.10 (s, 1H), 7.02 (dd, 1H, *J* = 7.5, 7.5 Hz), 6.97 (dd, 1H, *J* = 7.5, 7.5 Hz), 4.20 (s, 2H), 4.03 (s, 3H). ^{13}C-NMR (125 MHz, CD_3CN): δ_C 167.6, 137.1, 133.4, 127.8, 124.3, 123.1, 122.6, 121.8, 119.8, 119.1, 119.0, 114.8, 112.5, 111.7, 108.6, 65.6, 21.1. HREIMS *m/z* (% relative abundance) measured: 276.1262 (276.1262 calc. for $C_{18}H_{16}N_2O$. FTIR ν_{max}: 3441, 3056, 2927, 1695, 1609, 1456, 1345, 1221, 1091, 955, 741 cm^{-1}. UV (CH_2Cl_2) λ_{max} (log ε): 219 (4.5), 271 (3.8).

4.6.2.3. Synthesis of di-(1-methoxy-3-indolyl)-methane (120)



114 **120**

Fig **43**. Synthesis of di-(1-methoxy-3-indolyl)methane (**120)**.

To a solution of 1-methoxyindole (**114**, 30 mg, 0.20 mmol) dissolved in EtOH (1 ml), glacial acetic acid (10 µl, 0.17 mmol) was added to the solution and stirred at 50°C for 20 min. Then 40% formaldehyde (10 µl) was added to the reaction mixture and stirred at 90°C. After 3 hours, the reaction mixture was cooled and the solvent was

evaporated. The crude reaction mixture was subjected to reverse phase column chromatography (CH_3CN:H_2O-50:50) to afford di-(1-methoxy-3-indolyl)-methane (**120**, 8 mg, 0.05 mmol, 25% yield, Fig **43**, adapted from Jackson *et al*., 1987).

HPLC: R_T = 33.3 min. ^{1}H-NMR (500 MHz, CD_3CN): δ_H 7.58 (d, 2H, *J* = 7.5 Hz), 7.42 (d, 2H, *J* = 8 Hz), 7.25 (s, 2H), 7.21 (dd, 2H, *J* = 7.5, 8 Hz), 7.05 (dd, 2H, *J* = 7.5, 8 Hz), 4.17 (s, 2H) 4.04 (s, 6H). ^{13}C-NMR (125 MHz, CD_3CN): δ_C 133.3, 124.2, 122.7, 122.2, 119.8, 119.7, 111.8, 108.6, 65.7, 20.9. HREIMS *m/z* (% relative abundance) measured: 306.1367 (306.1368 calc. for $C_{19}H_{18}N_2O_2$). EIMS *m/z* (% relative abundance): 306 [M^+] (90), 275 (30), 243 (100), 149 (58).

4.6.2.4. Synthesis of brussalexin B (117)

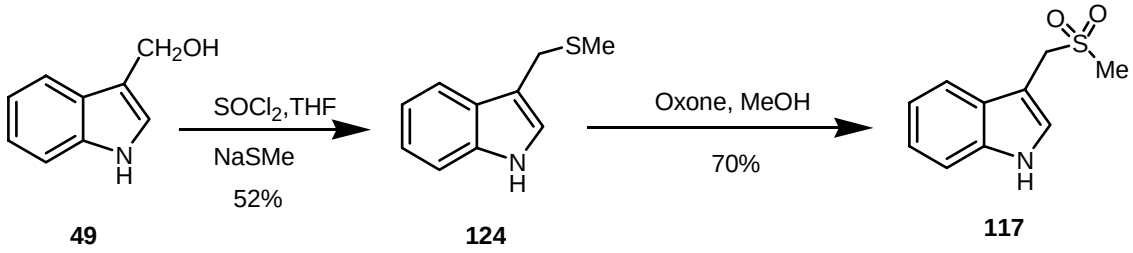


Fig **30**. Synthesis of brussalexin B (**117)**.

Indolyl-3-methylsulfanylmethane **(124)**

A mixture of indolyl-3-methanol (**49**, 200 mg, 1.36 mmol) and Et_3N (800 µl) in THF (4 ml) was cooled to 0°C; $SOCl_2$ (240 µl, 3.44 mmol) was added dropwise to the

above mixture and the reaction mixture was stirred for 50 min. Then the reaction mixture was concentrated and the residue was redissolved in THF (4 ml). After 10 min, NaSMe (264 mg 3.44 mmol) was added to the reaction mixture and stirred for 2 hours. The reaction mixture was diluted with water (10 ml) and extracted with Et_2O (15 ml × 3). The combined organic portion was dried over Na_2SO_4 and solvent was evaporated. The crude reaction mixture was subjected to flash column chromatography (EtOAc:hexane, 20:80) to afford 3-methylsulfanylmethylindole (**124**, 100 mg, 42%), m. p. 77-79 °C

HPLC: R_T = 19.3 min. ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H 7.70 (d, *J* = 8 Hz, 2H), 7.34(dd, *J* = 7.5, 7.5 Hz, 1H), 7.24 (dd, *J* = 8, 8 Hz, 2H), 7.06 (s, 1H), 3.85 (s, 2H), 2.68 (s, 3H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ_C 136.9, 127.3, 123.3, 122.9, 120.3, 119.6, 112.8, 111.9, 34.8, 15.7. FTIR ν_{max}: 3390, 3261, 2927, 1617, 1507, 1448, 1348, 1256, 1100, 940 cm^{-1}. UV (CH_2Cl_2) λ_{max} (log ε): 201 (4.4), 222 (4.4), 280 (3.8).

***Brussalexin B* (117)**

To a solution of 3-methylsulfanylmethylindole (**124**, 16 mg, 0.09 mmol) in MeOH (500 µl), oxone (176 mg, 0.32 mmol) solution in water (500 µl) was added and stirred at room temperature. After 10 min, the reaction mixture was filtered and the solution was extracted with Et_2O (10 ml × 3). The combined organic extract was dried over anhydrous Na_2SO_4 and concentrated. The crude reaction mixture was subjected to flash column chromatography (CH_2Cl_2-MeOH, 90:10) to afford brussalexin B (**117**, 15 mg, 0.08 mmol, 85%, Fig **30,** adapted from Latxague *et al*., 1991). m. p. 150-152 °C.

HPLC: R_T = 7.2 min. ^{1}H-NMR (500 MHz, CD_3CN) δ 9.53 (bs, 1H), 7.71 (d, *J* = 8 Hz, 1H), 7.48 (d, *J* = 8 Hz, 1H), 7.39 (d, *J* = 2.6 Hz, 1H), 7.21 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.16 (dd, *J* = 7.5, 7.5 Hz, 1H), 4.50 (s, 2H), 2.79 (s, 3H). ^{13}C-NMR (125 MHz, CD_3CN): δ 136.7, 127.6, 127.0, 122.5, 120.1, 119.4, 112.1, 102.9, 51.4, 38.9. HREIMS *m/z* (% relative abundance) measured: 209.0509 (209.0510 calc. for $C_{10}H_{11}NO_2S$. FTIR ν_{max}: 3358, 2925, 2852, 1653, 1458, 1296, 1114, 966, 891, 745 cm^{-1}. UV (CH_2Cl_2) λ_{max} (log ε): 217 (4.4), 270 (3.7).

4.6.2.5. Synthesis of brussalexin C (118)

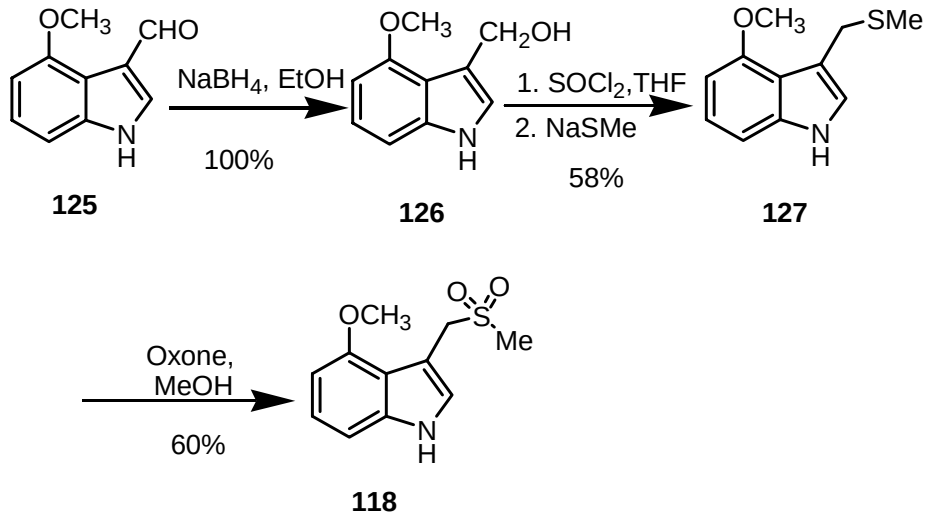


Fig **31**. Synthesis of brussalexin C (**118**).

***4-Methoxyindole-3-carboxaldehyde* (125)**

Indole-3-carboxaldehyde (**94,** 145 mg, 1.0 mmol) was added to a solution of thalium trifluoroacetic acid (815 mg, 1.8 mmol) and the mixture was stirred at room temperature. After 1.5 hours, trifluoroacetic acid was removed under reduced pressure and the residue was dissolved in DMF (2.5 ml); CuI (761 mg, 3.0 mmol) and I_2 (762 mg, 4.0 mmol) were added and the reaction mixture stirred for 1 hour at room temperature. Then MeONa solution (10 ml, 15%) was added and refluxed for 1 hour. The reaction mixture was diluted with CH_2Cl_2:MeOH, 95:5 (50 ml) and was filtered through celite. The filtrate was washed with water (5 ml × 3), dried over Na_2SO_4, and concentrated. The crude reaction mixture was subjected to flash column chromatography (EtOAc:hexane, 40:60) to afford 4-methoxyindole-3-carboxaldehyde (**125,** 102 mg, 58 %, Somei *et al*., 1984).

***4-Methoxyindole-3-methanol* (126)**

4-Methoxyindole-3-carboxaldehyde (**125**, 40 mg, 0.24 mmol) was dissolved in EtOH (1 ml) and cooled to 0°C. To the reaction mixture, $NaBH_4$ (30 mg, 0.90 mmol) was added and stirred for 30 min. The reaction mixture was concentrated, diluted with water (20 ml) and extracted with EtOAc (30 ml × 3). The combined organic extract was dried over Na_2SO_4 and the solvent was evaporated to give 4-methoxyindole-3-methanol (**126**, 40 mg, 100 % yield, adapted from Latxague *et al*., 1991).

^{1}H-NMR (500 MHz, CD_3CN) δ_H 8.23 (bs, 1H) 7.82 (d, J = 8 Hz, 1H), 7.43 (s, 1H), 7.28 (d, J = 8, 8 Hz, 1H), 6.73 (d, J = 8 Hz, 1H), 4.75 (s, 2H), 3.96 (s, 3H).

***4-Methoxy-3-methylsulfanylmethylindole* (127)**

A mixture of 4-methoxyindole-3-methanol (**126**, 40 mg, 0.23 mmol) in THF (1 ml) and Et_3N (150 µl) was cooled to 0°C, $SOCl_2$ (55 µl, 0.50 mmol) was added dropwise and the reaction mixture was stirred for 1 hour. The reaction mixture was concentrated, the residue was redissolved in THF (1 ml) and stirred for 10 min. Then NaSMe (60 mg, 0.78 mmol) was added and the reaction mixture stirred for 1 hour. The reaction mixture was diluted with water (10 ml) and extracted with Et_2O (15 ml × 3). The combined organic extract was dried over Na_2SO_4 and the solvent was removed. The crude reaction mixture was subjected to flash column chromatography (EtOAc:hexane, 20:80) to afford 4-methoxy-3-methylsulfanylmethylindole (**127**, 35 mg, 70 %, Fig **31**). m. p. 96-98 °C

^{1}H-NMR (500 MHz, CD_3CN) δ_H 7.06 (dd, J = 8, 8 Hz, 1H), 7.01 (d, J = 7.5 Hz, 1H), 6.50 (d, J = 8 Hz, 1H), 4.01(s, 2H), 3.90 (s, 3H), 2.08 (s, 3H). FTIR ν_{max}: 3390, 2927, 2854, 1617, 1507, 1448, 1348, 1256, 1100 cm^{-1}.

***Brussalexin C* (118)**

To a solution of 4-methoxy-3-methylsulfanylmethylindole (**127**, 30 mg, 0.15 mmol) in MeOH (2.5 ml), oxone (330 mg, 0.50 mmol) in water (2.5 ml) solution was added and the reaction mixture was stirred at room temperature. After 10 min, the reaction mixture was filtered and the filtrate was extracted with Et_2O (10 ml × 3). The combined organic extract was dried over anhydrous Na_2SO_4 and concentrated. The crude residue was subjected to flash column chromatography (CH_2Cl_2-MeOH, 90:10)

to afford brussalexin C (**118**, 20 mg, 66 % yield, Fig **31**, adapted from Hanquet & Lusinchi, 1993), m. p. 161-163 °C as a white solid.

HPLC: R_T = 8.7 min. ^{1}H-NMR (500 MHz, CD_3CN) δ_H 9.50 (bs, 1H), 7.27 (d, *J* = 2.5 Hz, 1H), 7.11(dd, *J* = 7.5, 7.5 Hz, 1H), 7.05 (d, *J* = 7.5 Hz, 1H), 6.60 (d, *J* = 7.5 Hz, 1 H), 4.77 (s, 2H), 4.64 (s, 3H), 2.70 (s, 3H). ^{13}C-NMR (125 MHz, CD_3CN): δ_C 137.8, 125.6, 123.5, 123.3, 116.9, 105.4, 102.5, 100.4, 55.0, 53.2, 38.6. HREIMS *m/z* (% relative abundance) measured: 239.0617 (239.0616 calc. for $C_{11}H_{13}NO_3S$). FTIR ν_{max}: 3358, 2922, 2841, 1653, 1509, 1350, 1291, 1253, 1113, 1081, 960, 890, 726 cm^{-1}. UV (CH_2Cl_2) λ_{max} (log ε): 220 (4.476), 268 (3.661).

3-Methanesulfinylmethyl-4-methoxyindole **(128)**

To a solution of 4-methoxy-3-methylsulfanylmethylindole (**127**, 6 mg, 0.03 mmol) in MeOH (500 µl), oxone (24 mg, 0.04 mmol) in water (500 µl) solution was added and stirred at room temperature. After 5 min, the reaction mixture was filtered and the filtrate was extracted with Et_2O (30 ml × 3). The combined organic extract was dried over anhydrous Na_2SO_4 and concentrated. The crude residue was subjected to flash column chromatography (CH_2Cl_2-MeOH, 90:10) to afford 2 mg of 3-methanesulfinylmethyl-4-methoxyindole (35 % yield, **128**).

^{1}H-NMR (500 MHz, CD_3CN) δ_H 9.37 (bs, 1H), 7.16 (d, 1H, *J* = 2.5Hz), 7.10 (dd, 1H, *J* = 8 Hz), 7.05 (d, 1H, *J* = 7.5Hz), 6.57 (d, 1 H, *J* = 7.5 Hz), 4.29 (s, 2H), 3.92 (s, 3H), 2.47 (s, 3H).

4.6.2.6. Synthesis of brussalexin A (121)

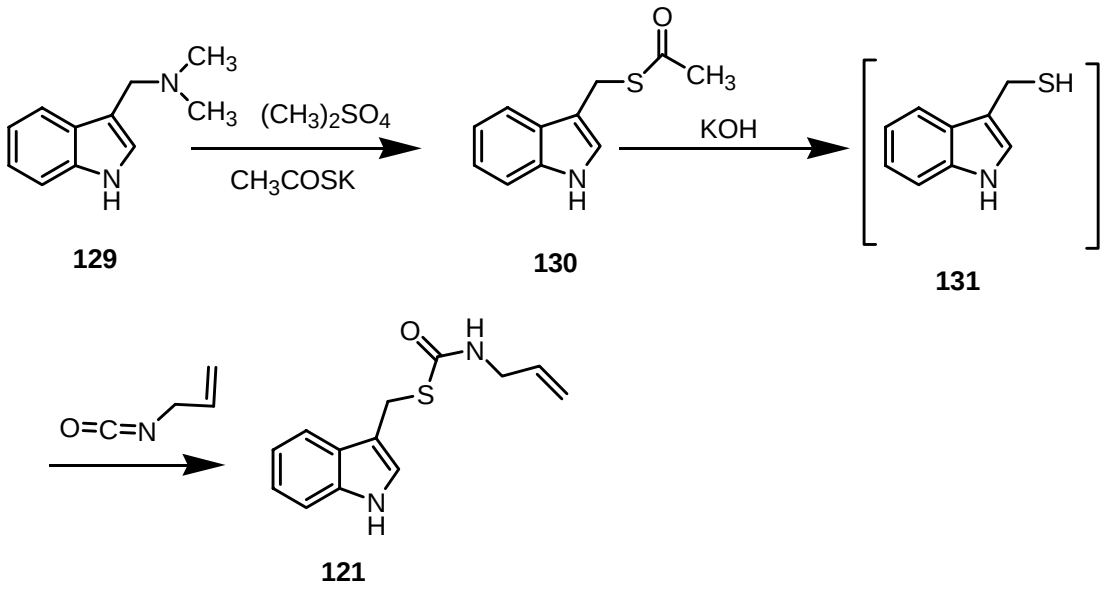


Fig **32**. Synthesis of Brussalexin A (**121**, overall 3 % yield)

S-3-Indolemethyl thioacetate (130)

To a solution of gramine (**129,** 116 mg, 0.66 mmol) and $(CH_3)_2SO_4$ (62 µl) in water (2 ml), a solution of CH_3COSH (65 µl) in KOH (1N, 1 ml) was added and the reaction mixture was refluxed for 20 min. After cooling to room temperature, the reaction mixture was extracted with Et_2O (5 ml × 3). The combined organic extract was dried over anhydrous Na_2SO_4 and concentrated. The crude reaction mixture was subjected to flash column chromatography (CH_2Cl_2:MeOH, 90:5) to afford *S*-3-

indolemethyl thioacetate (**130,** 110 mg, 81 % yield, Fig **32**) (Benghiat & Crooks, 1983).

HPLC R_T = 19.0 min. ^{1}H-NMR (500 MHz,CD_3CN): δ_H 8.26 (bs, 1H), 7.67 (d, *J* = 8 Hz, 1H), 7.37 (d, *J* = 8 Hz, 1H) 7.26 (dd, *J* = 8, 8 Hz, 1H), 7.21 (dd, *J* = 8, 8 Hz, 1H), 7.16 (d, *J* = 2.4 Hz, 1H), 4.15 (s, 1H), 2.39 (s, 1H). HREIMS *m/z* (% relative abundance) measured: 205.0557 (205.0561 calc. for $C_{11}H_{11}NOS$). UV (HPLC): λ_{max} 220, 285 nm.

***Brussalexin A* (121)**

S-3-Indolemethyl thioacetate (**130**, 29 mg, 0.14 mmol) in degassed THF (0.7 ml) was added to degassed KOH (1.4 ml, 1N) solution and stirred for 8 hours under argon atmosphere and then the solvent was removed under reduced pressure. The residue was redissolved in water with HCl (1N, 1.5 ml) and extracted with EtOAc (5 ml × 3). The combined extract was concentrated to a solid mass. The residue (24 mg) was redissolved in benzene (1 ml) and Et_3N (50 µl). Allylisocyanate (22 µl) was added to the solution and stirred for 12 hours. The reaction mixture was concentrated, and was subjected to preparative TLC (EtOAc:hexane, 20:80) to afford (3 mg) of brussalexin A (**121**, 3 % yield, adapted from Tsukamoto *et al.*, 2005).

HPLC R_T = 18.0 min. ^{1}H-NMR (500 MHz,CD_3CN): δ_H 9.17 (bs, 1H), 7.61 (d, *J* = 8 Hz, 1H), 7.41 (d, *J* = 8 Hz, 1H), 7.23 (d, *J* = 2.4 Hz, 1H), 7.17 (dd, *J* = 7, 8 Hz, 1H), 7.05 (dd, *J* = 8,7 Hz, 1H), 6.47 (bs, 1H), 5.81 (m, 1H), 5.17 (dd, *J* = 17, 1.5 Hz, 1H), 5.06 (dd, *J* = 11, 1.5 Hz, 1H), 4.34 (s, 2H), 3.85 (m, 1H). ^{1}H-NMR (500 MHz, $CDCl_3$):

δ_H 8.05 (bs, 1H), 7.69 (d, *J* = 8 Hz, 1H), 7.4 (d, *J* = 8 Hz, 1H), 7.2 (bs, 1H), 7.2 (dd, *J* = 7, 7.5 Hz, 1H), 7.18 (dd, *J* = 7.5 Hz, 1H), 5.85 (m, 1H), 5.4 (bs, 1H), 5,23 (d, *J* = 12 Hz, 1H), 5.17 (d, *J* = 12 Hz, 1H), 4.4 (s, 2H), 3.96 (bs, 1H). ^{13}C-NMR (125 MHz CD_3CN): δ_C 167.5, 136.3, 133.7, 126.6, 123.4, 122.4, 119.8, 119.0, 116.9, 112.3, 111.3, 43.7, 25.5. HREIMS *m/z* (% relative abundance) measured: 246.0825 (246.0826 calc. for $C_{13}H_{14}N_2OS$). FTIR ν_{max}: (KBr)/cm-^{1}: 3395, 3315, 3046, 2922, 1652, 1497, 1420, 1340, 1203, 743. λ_{max} (log ε): 228 (4.4), 279 (3.9).

4.7. Brown mustard

4.7.1. Isolation of metabolites from brown mustard (*Brassica juncea* var. Commercial Brown)

Brown mustard plants elicited with $CuCl_2$ (1×10^{-2} M) solution. After 36 hours, the leaves (800 gm) were harvested, crushed in liquid N_2 and extracted with MeOH (3 × 1500 ml). The crude extract was redissolved in water and EtOAc. The water soluble extract (49 g) was fractionated on reversed phase column chromatography (15 cm × 3 cm), eluted with a gradient of H_2O:CH_3CN. Sixteen fractions were collected and analysed by HPLC. F 5 showed a peak at R_T = 11.8 min (Albpol), F 6 showed a peak at R_T = 7.2 min, F 7 showed a peak at R_T = 14.0 min, F 8 showed a peak at R_T = 14.7 min and F 10 showed a peak at R_T = 21.0 min. L-Tryptophan (**86**) was isolated from F 5 by reverse phase column chromatography (CH_3CN:H_2O) and sephadex column

chromatography (MeOH:H_20, 95:5). Phenylalanine (**132**) was isolated from F 6 by reverse phase column chromatography (CH_3CN:H_2O). Compound **Y** was isolated from F 7 by reverse phase column chromatography (CH_3CN: H_2O). Isorhamnetin 3,7-diglucoside (**134**) was isolated from F 8 by reverse phase column chromatography (CH_3CN:H_2O). Spirobrassinin (**71**), indolyl-3-acetonitrile (**76**) and methylindole-3-carboxylate (**133**) were isolated from F 11 by reverse phase column chromatography (CH_3CN: H_2O).

Compound **Y**

HPLC: R_T = 14.4 min. ^{1}H-NMR (500 MHz, CD_3OD) δ_H 7.64 (d, *J* = 7.5 Hz, 1H), 7.39 (d, *J* = 8 Hz, 1H), 7.22 (dd, *J* = 7, 7.5 Hz, 1H), 7.12 (dd, *J* = 7, 7.5 Hz, 1H), 4.73 (bs, 1H), 4.58 (s, 1H), 3.90 (d, *J* = 11.5 Hz, 1H), 3.70 (bm, 2H), 3.69 (bs, 1H), 3.67 (s, 2H), 3.57 (dd, *J* = 5, 4.5 Hz, 1H), 3.20 (d, *J* = 9 Hz, 1H), 3.07 (m, 1H). 2.81 (bs, 1H) HREIMS *m/z* (% relative abundance) measured: 274 (61), 227 (41), 163 (100), 161 (37), 128 (39), 117 (58). UV (HPLC): λ_{max} 225, 285 nm.

Methylindole-3-carboxylate (**133**)

HPLC: R_T = 21.0 min (Albpol). ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H 9.1 (bs, 1H), 8.16 (d, 1H, *J* = 8 Hz), 7.96 (s, 1H), 7.46 (d, 1H, *J* = 8 Hz), 7.29 (m, 2H), 7.22 (s, 1H), 3.90 (s, 3H).

Isorhamnetin 3,7-diglucoside (**134**)

HPLC R_T = 14.7 min. UV (HPLC): λ_{max} 205, 245 nm. ^{1}H-NMR (500 MHz, CD_3OD) δ_H 7.95 (d, *J* = 2.0 Hz, 1H), 7.65 (d, *J* = 2.0 Hz, 1H), 7.63 (d, *J* = 2.0 Hz, 1H), 6.94 (s, 1H), 6.92 (s, 1H), 6.80 (d, *J* = 2.0 Hz, 1H), 6.51 (d, *J* = 2.0 Hz, 1H), 5.46 (d, *J* = 7.5 Hz, 1H), 5.08 (d, *J* = 7.0 Hz, 1H), 3.92 (s, 1H), 3.91 (s, 1H), 3.76 (m, 3H), 3.55 (m, 2H), 3.45 (m, 4H), 3.41 (m, 1H), 3.13 (m, 1H). ^{13}C-NMR (CD_3CN): δ_C 178.6, 163.8, 161.8, 158.3, 157.0, 150.2, 147.5, 134.6, 123.1, 121.9, 115.1, 113.6, 106.6, 102.5, 100.7, 99.9, 94.9, 76.9, 74.9, 73.8, 70.6, 70.3, 61.6, 61.5 and 55.93.

4.8. Wild species: Isolation of metabolites

4.8.1. Metabolites from Asian mustard (*Brassica tournefortii*)

Asian mustard plants were sprayed with $CuCl_2$ solution (1×10^{-2} M). The elicited leaves were harvested after 96 hours, frozen with liquid N_2, powdered with a glass rod and extracted with EtOAc (12 hours). The EtOAc was filtered, the filtrate was dried over Na_2SO_4 and the solvent was evaporated to dryness to yield a residue (1.2 g). The residue was chromatographed on flash column chromatograph (15 cm × 5 cm) eluted with a gradient mixture of CH_2Cl_2:hexane followed by CH_2Cl_2:MeOH. Thirteen fractions were collected and analysed by HPLC. F 12 showed a peak at R_T = 4.5 min which was isolated after reverse phase column chromatography (CD_3CN: H_2O) and flash column chromatography (CH_2Cl_2:hexane) to yield 3-methanesulfinyl propylisothiocyanate (**135**).

3-methanesulfinyl propylisothiocyanate **(135)**

HPLC R_T = 4.5 min. UV (HPLC): λ_{max} 205, 245 nm. Optical rotation: $[\alpha]_D^{24.5}$ -65.8, (c 4.8, CH_2Cl_2). ^{1}H-NMR (500 MHz, $CHCl_3$) δ_H 3.75 (dd, 2H, *J* = 6.5, 6.5 Hz), 2.82 (m, 1H), 2.71 (m, 1H), 2.54 (s, 3H), 2.10 (m, 2H). ^{13}C-NMR ($CHCl_3$): δ_C 129.8, 50.8, 44.5, 38.5 and 23.3. EIMS: 163 (6%, M^+), 71 (100%). IR: ν^{Film} 3427, 2933, 2183, 2099, 1444, 1349, 1046, and 1016 cm^{-1}.

4.8.2. Metabolites from sand rocket (*Diplotaxis tenuifolia*)

Plants (4-week old) were sprayed with $CuCl_2$ solution (1×10^{-2} M). The elicited leaves were harvested after 72 hours, frozen with liquide N_2, powdered with glass rods, and extracted with EtOAc for next 12 hours. The crushed leaves in EtOAc were filtered, the filtrate was dried over Na_2SO_4 and the solvent was evaporated to dryness to yield a residue (1.5 g). The residue was chromatographed on silica gel column (15 cm × 5 cm) eluted with a gradient of CH_2Cl_2:hexane, CH_2Cl_2:MeOH. F 4 was further subjected to reverse phase column chromatography, guided by bioassay against *C. cucumerinum* to isolate 5-(3-isothiocyanato-propylsulfanyl)-pentylisothiocyanate (**136**) and arvelexin (**84**) having retention time at R_T = 14.0 and 22.9 min.

5-(3-isothiocyanato-propylsulfanyl)-pentylisothiocyanate **(136)**

HPLC R_T = 22.9 min. ^{1}H-NMR (500 MHz, CD_3CN) δ_H 3.63 (t, *J* = 3.0 Hz, 2H), 2.76 (t, *J* = 7.0 Hz, 4H), 2.44 (t, *J* = 7.0 Hz, 2H), 1.80 (m, 6H) 1.73 (m, 2H). ^{13}C-NMR ($CHCl_3$): δ_C 119.7, 45.1, 29.0, 28.9, 28.3, 26.5, 24.5, 24.4 and 17.3. EIMS *m/z* (%

relative abundance) measured: 260 (12), 114 (100), 71(61), HREIMS: m/z 260.0472 (12%, M^+), cal. 260.04756 calc. for $C_{10}H_{16}N_2S_3$. UV (HPLC): λ_{max} 215, 260 nm.

4.8.3. Metabolites from wallrocket (*Diplotaxis muralis*)

Plants (4-week old) were sprayed with $CuCl_2$ solution (1×10^{-2} M) and incubated for 2 days. Leaves (3.2 kg) were harvested, frozen in liquid N_2, crushed, and extracted with EtOAc for 12 hours. The EtOAc extract was dried over anhydrous Na_2SO_4, concentrated to dryness. The residue (5.7 g) was chromatographed on silica gel column (15 cm × 5 cm) and eluted with a gradient mixture of CH_2Cl_2:hexane and CH_2Cl_2:MeOH. Ten fractions were collected and analysed by HPLC. F 4 contained arvelexin (**84**) having peak at R_T = 13.9 min, F 5 showed a peak at R_T = 28.0 min, F 6 showed a peak at R_T = 36.1 min, F 7 showed a peak at R_T = 20.1 min and F 8 showed a peak at R_T = 24.0 min. F 6 was subjected to reverse phase column chromatography (CH_3CN: H_2O) to yield bis (isothiocyanatobutyl) disulfide (**139**, 8.8 mg). Rapalexin A (**138**, 1 mg) was isolated from F 7 by flash column chromatography (Et_2O:hexane) and reverse phase column chromatography (CH_3CN:H_2O). 1,4-Dimethoxyindole-3-acetonitrile (**137**, 2 mg) was isolated from F 8 by flash column chromatography (Et_2O:hexane) and reverse phase column chromatography (CH_3CN:H_2O).

1, 4-Dimethoxyindole-3-acetonitrile (**137**)

HPLC R_T = 20.1 min. ^{1}H-NMR (500 MHz, CD_3CN): δ_H 7.32 (s, 1H), 7.20 (dd, *J* = 8, 8 Hz, 1H), 7.05 (d, *J* = 8 Hz, 1H), 6.61 (d, *J* = 7.5 Hz, 1H), 4.07 (s, 3H), 4.00 (s, 2H), 3.93 (s, 3H). HREIMS: *m/z* 216.0898 (81%, M^+), cal. 216.0898 for $C_{12}H_{12}N_2O_2$, EIMS: *m/z* 216 (81%) 185 (100%).

Rapalexin A **(138)**

HPLC R_T = 24.0 min. ^{1}H-NMR (500 MHz, CD_3CN): δ_H 9.38 (bs, 1H), 7.20 (d, *J* = 3 Hz, 1H), 7.17 (dd, *J* = 8, 8 Hz, 1H), 7.06 (d, *J* = 8 Hz, 1H), 6.64 (d, *J* = 8 Hz, 1H), 3.93 (s, 3H). HREIMS: *m/z* 204.0349 (100%, M^+), cal. 204.0357, 189.0123 (100%).

4-Methoxyindole-3-thiocyanate **(141)**

R_T = 26.1 min. ^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 8.54 (brs, 1H), 7.51 (d, *J* = 3.8 Hz, 1H), 7.13 (d, *J* = 8.7 Hz, 1H), 7.02 (d, *J* = 8.7 Hz, 1H), 5.53 (brs, 1H), 4.09 (s, 3H). ^{1}H NMR (500.1 MHz, CD_3OD): δ 7.56 (s, 1H), 7.08 (d, *J* = 8.65 Hz, 1H), 6.88 (d, *J* = 8.65 Hz, 1H), 4.01 (s, 3H). HREI-MS: calc. for $C_{10}H_8N_2O_2S$, m/z 220.0306, found 220.0313.

bis (Isothiocyanatobutyl) disulfide **(139)**

HPLC R_T = 28.0 min. ^{1}H-NMR (500 MHz, CD_3CN): δ_H 3.58 (dd, *J* = 6.5, 6.5 Hz, 4H), 2.73 (dd, *J* = 6.5, 6.5 Hz, 4H), 1.85 (m, 4H), 1.85 (m, 4H). ^{13}C-NMR (125 MHz,

CD_3CN): δ_c 130.4, 44.7, 37.8, 28.6, 26.1. HR-MS: m/z 292.0083 (15%), Cal. 292.007532 calc. for $C_{10}H_{16}N_2S_4$.

4.8.4. Metabolites from hedge mustard (*Sisymbrium officinale*)

Plants (4-week old, 100) were sprayed with $CuCl_2$ (1×10^{-2} M) and incubated for two days. Elicited (200 g) leaves were collected separately, frozen in liquid N_2, crushed and extracted with EtOAc (300 ml × 3). The EtOAc extract was filtered, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue (2.8 g) was chromatographed on a silica gel column (15 cm × 5 cm), eluted with a gradient mixture of (C_6H_{12}:CH_2Cl_2:MeOH). F 6 showed a peak at R_T 11.8 min and F 7 showed a peak at R_T 19.0 min. Indolyl-3-acetonitrile (**76**) was isolated from F 6 by reverse phase column chromatography and methyl 1-methoxyindole-3-carboxylate (**142**) was isolated from F 7 by reversed column chromatography (CH_3CN:H_2O).

Methyl 1-methoxyindole-3-carboxylate **(142)**

HPLC: R_T = 18.0 min. ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H 8.16 (d, 1H, *J* = 8 Hz), 7.96 (s, 1H), 7.46 (d, 1H, *J* = 8 Hz), 7.29 (m, 2H), 7.22 (s, 1H), 4.14 (s, 3H), 3.90 (s, 3H). ^{13}C-NMR (125 MHz, CD_3CN): δ_C 165.2, 132.2, 128.5, 123.7, 123.0, 122.6, 122.1, 108.8, 103.7, 66.8, 51.3. HREIMS m/z (% relative abundance) measured: 205.0736 (205.0739 calc. for $C_{11}H_{11}NO_3$.

4.8.5 Metabolites from Abyssinian mustard (*Crambe abyssinica)*

Plants (3-week old) were elicited by sprayed with $CuCl_2$ solution (1×10^{-2} M). Elicited leaves were collected, frozen in liquid nitrogen, crushed, and extracted with EtOAc. The crude extract (1.6 g) was fractionated by gradient flash column chromatography (C_6H_{14} : CH_2Cl_2 : MeOH). Fraction F 7 was further subjected to multiple column chromatography and reverse phase column chromatography (CH_3CN: H_2O) to yield the arvelexin (**84**) and rapalexin B (**143**) with retention times at R_T = 13.9 and 15.0 min respectively.

Rapalexin B **(143)**

R_T = 15.0 min.

^{1}H-NMR (500 MHz, $CDCl_3$): δ_H 8.54 (brs, 1H), 7.51 (d, *J* = 3.8 Hz, 1H), 7.13 (d, *J* = 8.7 Hz, 1H), 7.02 (d, *J* = 8.7 Hz, 1H), 5.53 (brs, 1H), 4.09 (s, 3H). 1H-NMR (500.1 MHz, CD_3OD): δ 7.56 (s, 1H), 7.08 (d, *J* = 8.65 Hz, 1H), 6.88 (d, *J* = 8.65 Hz, 1H), 4.01 (s, 3H). ^{13}C-NMR (125 MHz, $CDCl_3$): δ_C 144.2 (s), 138.9 (s), 132.7 (s), 132.5 (d), 114.5 (d), 112.9 (s), 112.6 (s), 109.0 (d), 63.6 (q). HREI-MS: m/z 220.0313. calc. 220.0306 for $C_{10}H_8N_2O_2S$.

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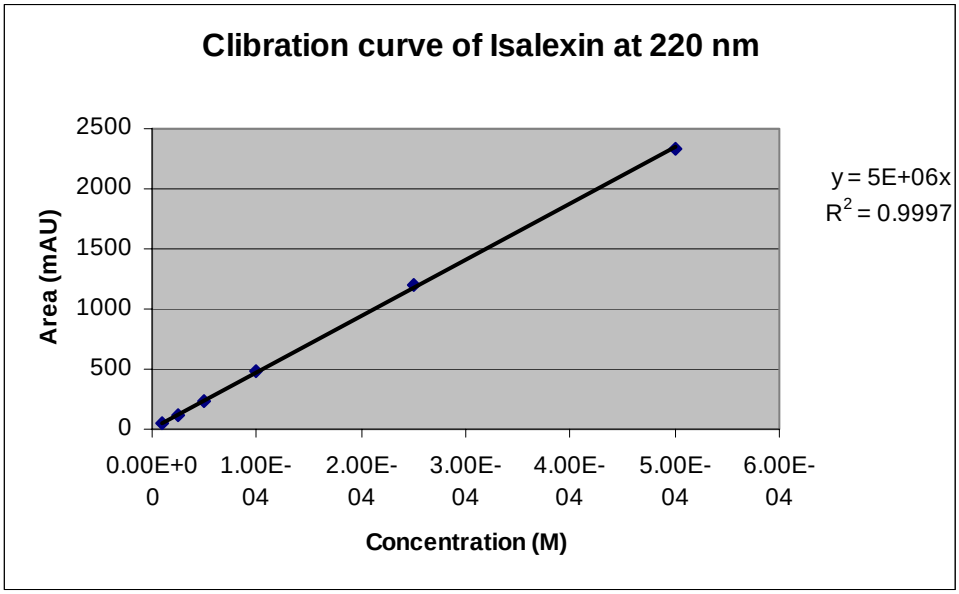
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Concentration (M)
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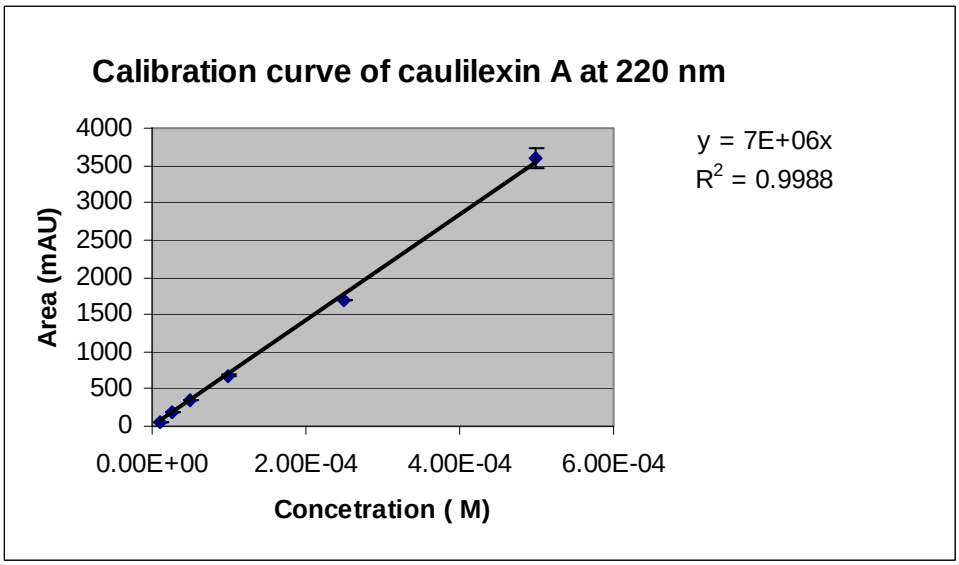
R2 = 0.9997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500
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Concetration (M)
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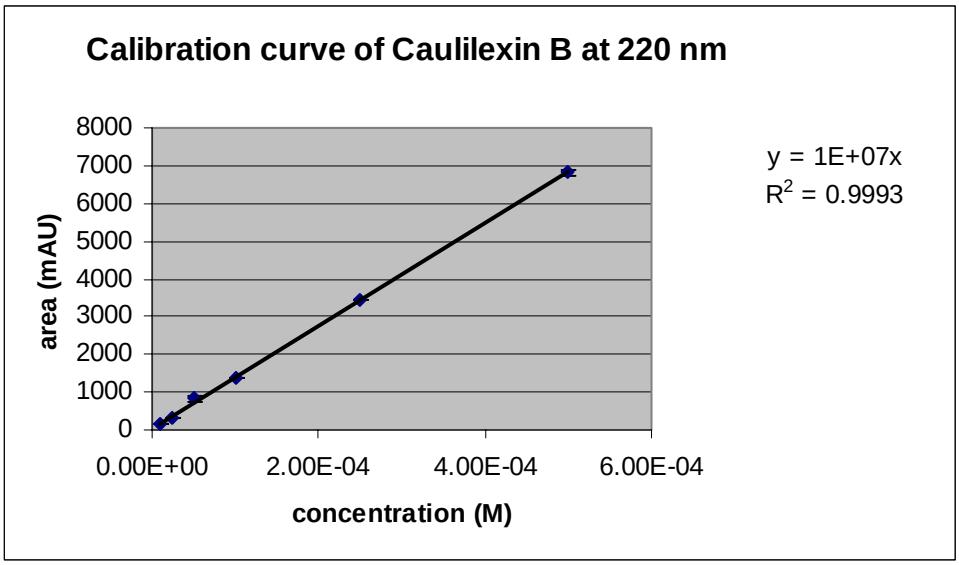
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2000
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concentration (M)
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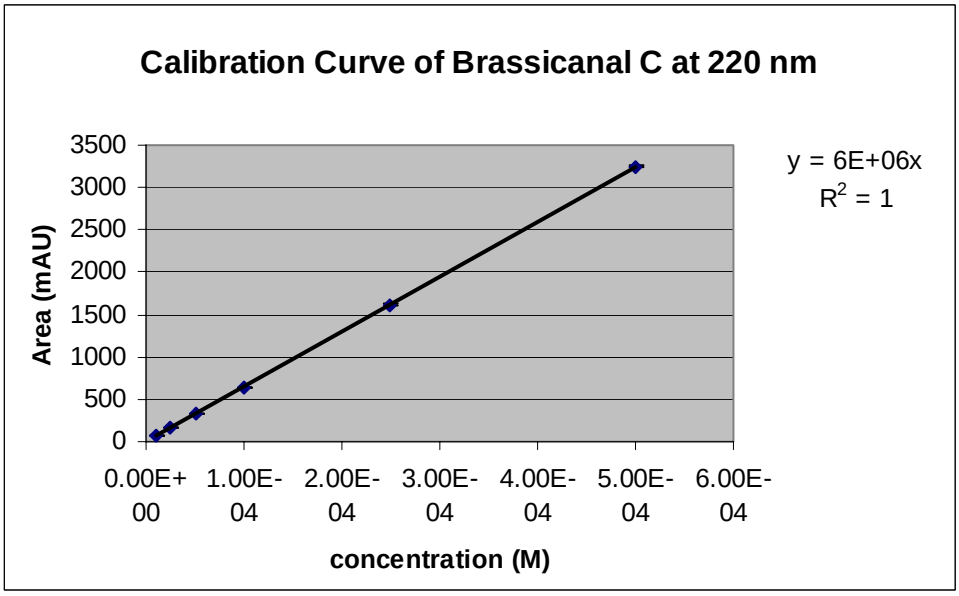
R2 = 0.9993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6.00E-04
concentration (M)
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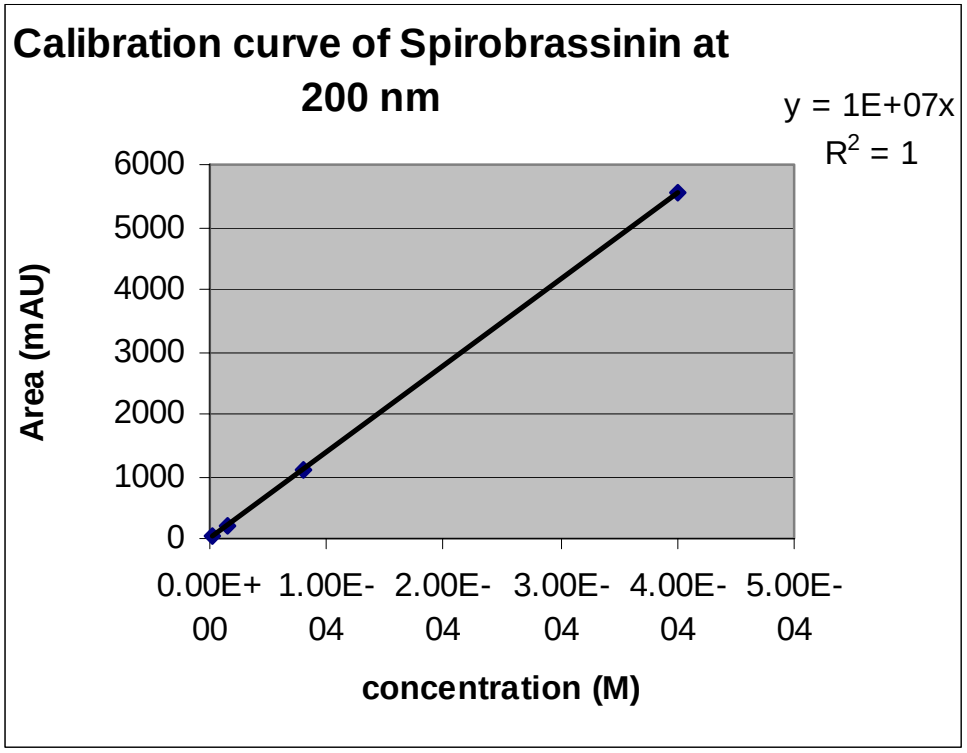
R2 = 1


Calibration curve of Spirobrassinin at 200 nm
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R2 = 1
Area (mAU)
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5000
4000
3000
2000
1000
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1.00E-04
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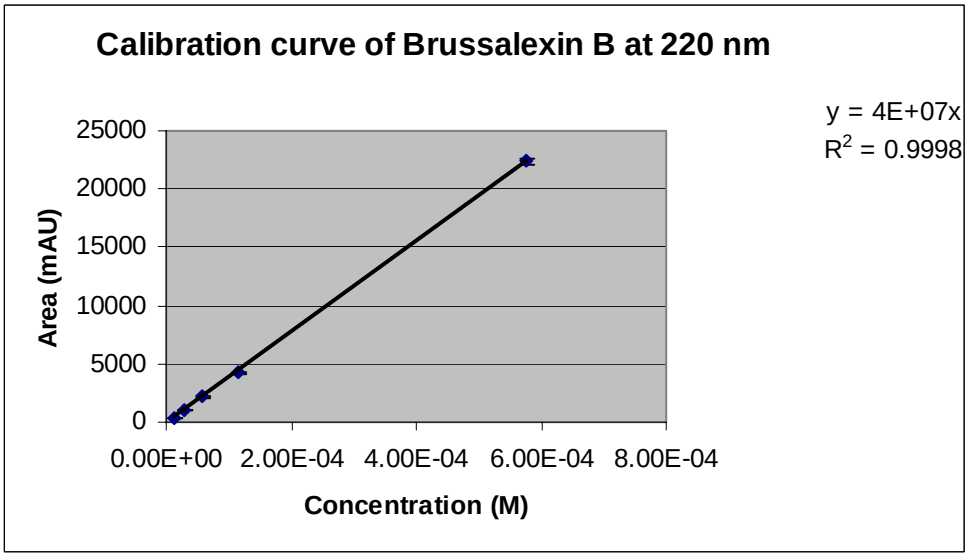
3.00E-04
4.00E-04
5.00E-04
concentration (M)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R2 = 0.9998
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20000
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2.00E-04
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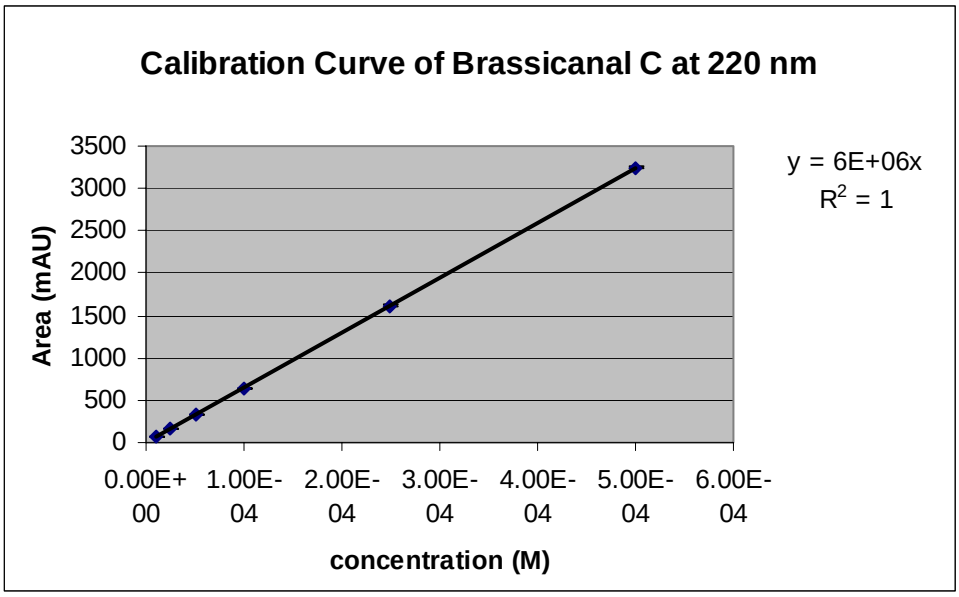
6.00E-04
8.00E-04
Concentration (M)


Calibration Curve of Brassicanal C at 220 nm
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R2 = 1
Area (mAU)
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2500
2000
1500
1000
500
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0.00E+00
1.00E-04
2.00E-04
3.00E-04
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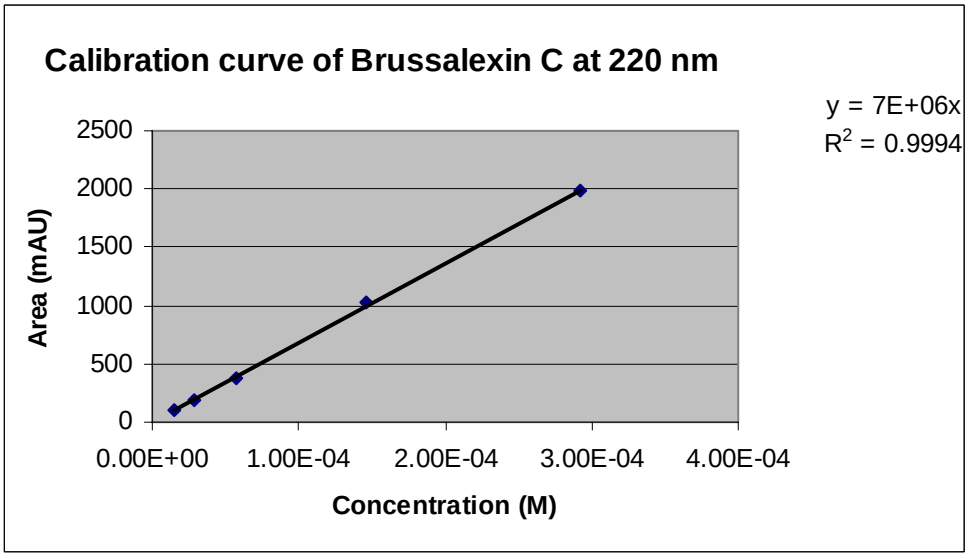
5.00E-04
6.00E-04
concentration (M)


Calibration curve of Brussalexin C at 220 nm
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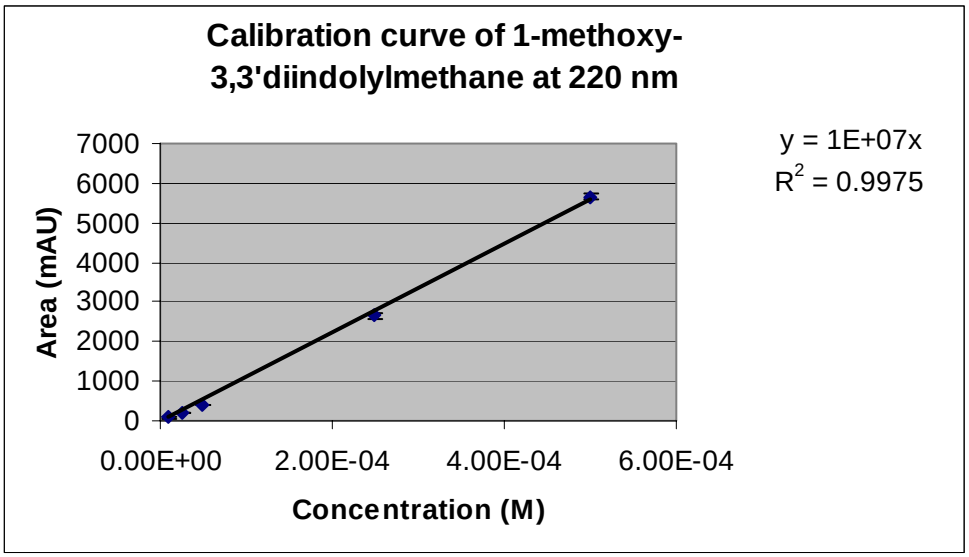
3.00E-04
4.00E-04
Concentration (M)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R2 = 0.9975
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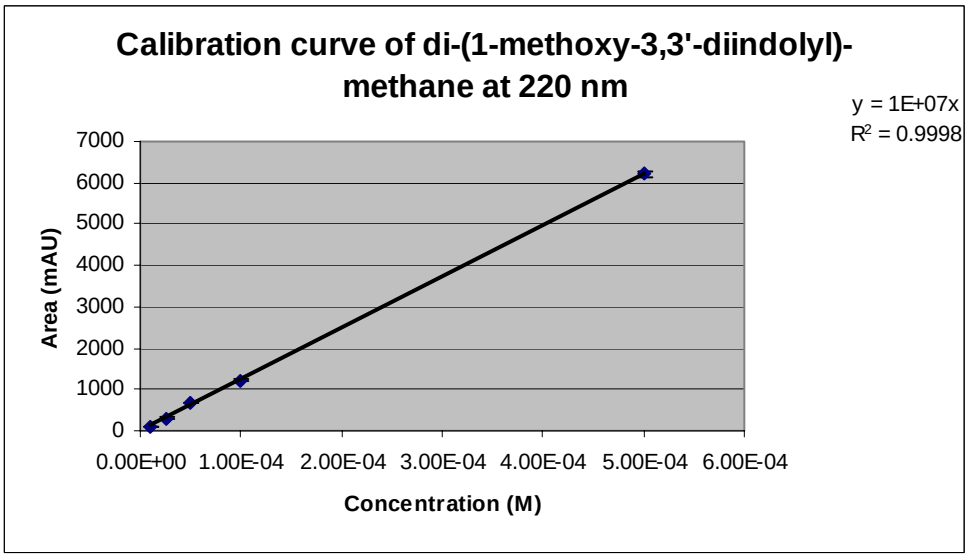
Concentration (M)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R2 = 0.9998
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5000
4000
3000
2000
1000
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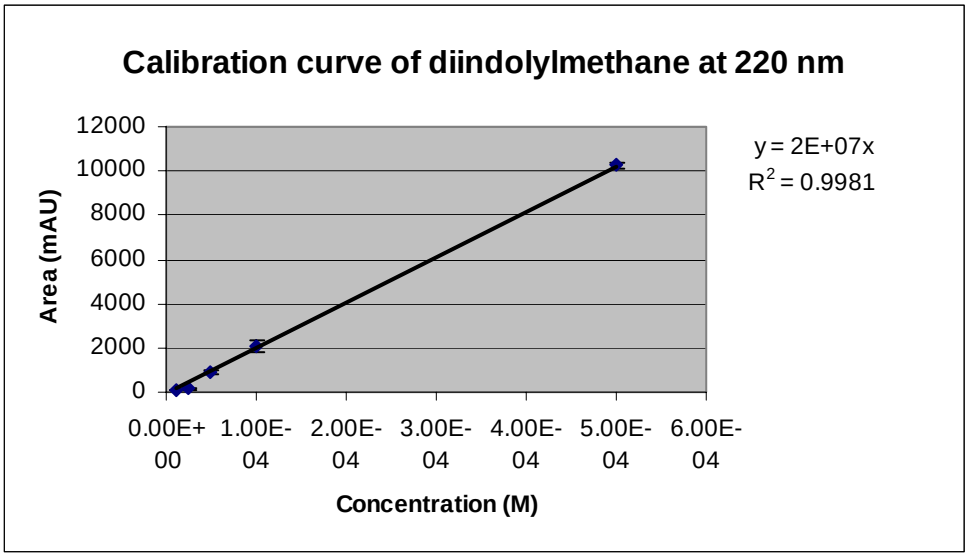
Concentration (M)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Concentration (M)
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R2 = 0.9981