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Phytoalexins from crucifers: synthesis, biosynthesis, and biotransformation

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Abstract

Phytoalexins play a significant role in the defense response of plants. These secondary metabolites, which are synthesized de novo in response to diverse forms of stress, including fungal infection, are part of the plants' chemical and biochemical defense mechanisms. Phytoalexins from crucifers are structurally and biogenetically related, but display significantly different biological activities. Here, we review work reporting the chemical structures, synthesis, biosynthesis and metabolism of cruciferous phytoalexins, as well as their biological activity towards different microorganisms. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Biosynthesis; Brassica; Crucifer; Cruciferae; Metabolism; Phytoalexin; Synthesis

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1. Introduction

Similar to other plants (Keen, 1993; Staskawicz, Ausubel, Baker, Ellis & Jones, 1995; Hammond-Kosac & Jones, 1996; Osbourn, 1996), the disease resistance of crucifers is related with both constitutive and induced defenses. Phytoalexins are part of the induced chemical defenses produced by plants in response to several forms of stress, including microbial attack (Bailey & Mansfield, 1982; Brooks & Watson, 1985). In general, the timing, rate of accumulation, and rela-

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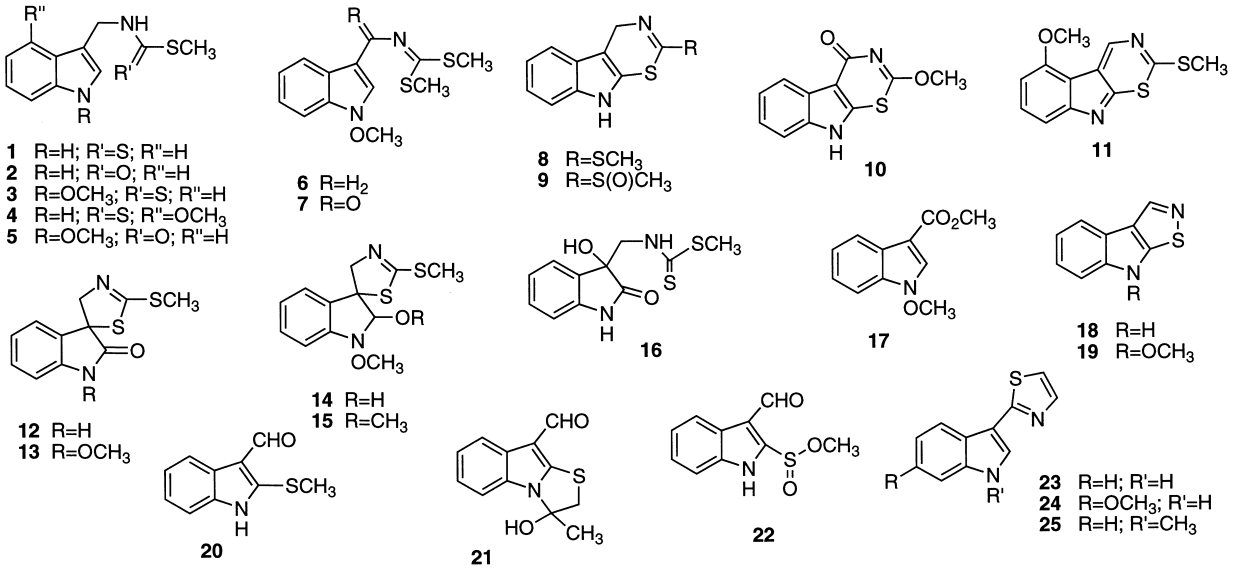


Fig. 1. Structures of cruciferous phytoalexins: **1** brassinin, **2** brassitin, **3** 1-methoxybrassinin, **4** 4-methoxybrassinin, **5** 1-methoxybrassitin, **6** 1-methoxybrassenin A, **7** 1-methoxybrassenin B, **8** cyclobrassinin, **9** cyclobrassinin sulfoxide, **10** cyclobrassinone, **11** dehydro-4-methoxycyclobrassinin, **12** spirobrassinin, **13** 1-methoxyspirobrassinin, **14** 1-methoxyspirobrassinol, **15** 1-methoxyspirobrassinol methyl ether, **16** dioxibrassinin, **17** methyl 1-methoxyinodole-3-carboxylate, **18** brassilexin, **19** sinalexin, **20** brassicanal A, **21** brassicanal B, **22** brassicanal C, **23** camalexin, **24** 6-methoxycamalexin, **25** 1-methylcamalexin.

tive amounts of phytoalexins play significant roles in plant resistance to pathogen invasion (Dixon, 1986; Dixon, Harrison & Lamb, 1994; Kúc, 1995; Smith, 1996). Although phytoalexins from over 30 different plant families have been isolated since Muller (Deverall, 1982; Müller, 1958) first proposed this term in 1940, cruciferous phytoalexins were the first reported sulfur-containing phytoalexins. Glucosinolates are also sulfur- containing metabolites produced by crucifers and thought to be part of the crucifer constitutive defenses (Fenwick, Heaney & Mawson, 1989; Giamoustaris & Mithen, 1997). Interestingly, and perhaps justifiably, the cruciferous family (Cruciferae syn. *Brassicaceae*) has high sulfur requirements (Marquard & Walker, 1995).

Cruciferous crops are cultivated worldwide and constitute an extremely valuable group of plants. Enormous quantities of vegetable crucifers, such as broccoli (*Brassica oleracea* var. *botrytis*), cauliflower (*B. oleracea* var. *italica*), kale (*B. oleracea* var. *acephala*), radish (*Raphanus sativus*), and a variety of cabbages (*B. oleracea*) are consumed annually (Kimber & McGregor, 1995). Oilseed crucifers (Brassica spp.) constitute the third largest source of edible vegetable oils annually (Kimber & McGregor, 1995) and brown (*B. juncea*) and white (*Sinapis alba*) mustard seeds (Hemingway, 1995), as well as wasabi (*Wasabiae japonica*) (Chadwick, Lumpkin & Elberson, 1993) are well-known condiments. In fact, the worldwide impact of cruciferous crops is best assessed by the tremendous number and variety of scientific articles published annually.

2. Chemical structures, elicitation, and biological activity

Phytoalexins from crucifers were first reported in 1986 by Takasugi and co-workers (Takasugi, Katsui, & Shirata, 1986). Since then, although a relatively larger number of cruciferous species remain to be investigated, close to thirty cruciferous phytoalexins have been isolated and their structures elucidated (Fig. 1, compounds **1**–**25**). Most interestingly, crucifers appear to be the only plant family producing sulfur-containing phytoalexins. Several of these phytoalexins are produced by more than one species and can be elicited by diverse pathogens and/or abiotic factors, as shown in Table 1. Brassinin (**1**) and 1-methoxybrassinin (**3**), which contain a dithiocarbamate group attached to a 3-methylindolyl moiety, and cyclobrassinin (**8**) were the first brassica phytoalexins to be reported (Fig. 1) (Takasugi et al., 1986). It is worthy to note that, although dithiocarbamates have long been known as important pesticides and herbicides (Thorn & Ludwig, 1962), so far crucifers appear to be the only plants producing these compounds. Methyl 1-methoxyindole-3-carboxylate (**17**) appears to be the first non-sulfur-containing phytoalexin isolated from a crucifer (Pedras & Sorensen, 1998). A compound similar to **17**, methyl

Table 1
Phytoalexins from crucifers, inducing agent, and biological activity

Phytoalexin (#) (reference for first isolation)	Plant species (inducing agent, reference)	Antimicrobial activity (reference)
Brassicanal A (**20**) (Monde et al., 1990a).	*Brassica oleracea* (*Pseudomonas cichorii*, Monde et al., 1991a).	*Bipolaris leersiae* (Monde et al., 1990a, 1990b).
	B. rapa syn. *B. campestris* (*P. cichorii*, Monde et al., 1990a)	*Phoma lingam* (Pedras & Khan, 1996; Pedras et al., 1997b)
	Raphanus sativus (*P. cichorii*, Monde et al., 1995).	
Brassicanal B (**21**) (Monde et al., 1990a, 1990b).	*B. rapa* (*P. cichorii*, Monde et al., 1990a, 1990b).	*B. leersiae* (Monde et al., 1990a, 1990b).
Brassicanal C (**22**) (Monde, Sasaki, Shirata & Takasugi, 1991b).	*B. oleracea* (*P. cichorii*, Monde et al., 1991a, 1991b).	*B. leersiae* (Monde et al., 1991b).
Brassilexin (**18**) (Devys et al., 1988)	*B. carinata* (abiotic — $CuCl_2$, *P. lingam*, Rouxel, Kollman, Boulidard & Mithen, 1991; Storck & Sacristan, 1995).	*P. lingam* (Pedras & Okanga, 1999; Pedras et al., 1997b).
	B. juncea (abiotic — $CuCl_2$, *Alternaria brassicae*, *P. lingam*, (Devys et al., 1988; Rouxel et al., 1991; Storck & Sacristan, 1995). *B. napus* (abiotic — $CuCl_2$, *P. lingam*, Rouxel et al., 1991; Storck & Sacristan, 1995). *B. nigra* (abiotic — $CuCl_2$, Rouxel et al., 1991) *B. oleracea* (abiotic — $CuCl_2$, Rouxel et al., 1991). *B. rapa* (abiotic — $CuCl_2$, Rouxel et al., 1991) *Sinapis arvensis* (*P. lingam*, Storck & Sacristan, 1995).	
Brassinin (**1**) (Takasugi et al., 1986).	*B. napus* (abiotic — $CuCl_2$, Rouxel et al., 1991).	*B. leersiae, Pyricularia orizae* (Takasugi et al., 1986, 1988).
	B. oleracea (*P. cichorii*, Monde et al., 1991a). *B. rapa* (abiotic — UV light, *Erwinia carotovora, P. cichorii*, Takasugi et al., 1986, Takasugi et al., 1988; Monde et al., 1994b). *R. sativus* (*P. cichorii*, Monde et al., 1995).	*P. lingam* (Pedras et al., 1992).
Brassitin (**2**) (Monde et al., 1995).	*R. sativus* (*P. cichorii*, Monde et al., 1995).	*B. leersiae* (Monde et al., 1995).
Camalexin (**23**) (Browne et al., 1991).	*A. thaliana* (*P. syringae*, Tsuji, Zook, Jackson, Cage, Hammerschmidt & Somerville, 1992).	*A. brassicae, Cladosporium sp.* (Jimenez et al., 1997; Browne et al., 1991)
	Arabis lyrate (*Cochliobolus carhonum*, *P. syringae*, Zook, Leege, Jackson & Hammerschmidt, 1998).	*Bacillus subtilis, Escherichia coli, Fusarium oxysporum, Listeria monocytogenes, P. syringae, Saccharomyces cerevisiae, Xanthomonas campestris* (Rogers, Glazebrook & Ausubel, 1996).
	Capsella bursa-pastoris (*A. brassicae*, Jimenez et al., 1997).	*C. cucumerinum, P. syringae* (Tsuji et al., 1992). *E. carotovora, P. cichorii, P. lingam, P.syringae, R. solani, X. campestris* (Pedras et al., 1998a). *Rhizoctonia solani* (Conn, Browne, Tewari & Ayer, 1994).
Cyclobrassinin (**8**), (Takasugi et al., 1986).	*B. carinata* (abiotic — $CuCl_2$, Rouxel et al., 1991).	*B. leersiae, P. orizae* (Takasugi et al., 1986, 1988)
	B. juncea (abiotic — $CuCl_2$, Rouxel et al., 1991). *B. napus* (*P. lingam*, Dahyia & Rimmer, 1988).	*A. brassicae, Botrytis cinerea, C. cucumerinum, F. nivale, P. lingam, Pythium ultimum, R. solani, Sclerotinia sclerotiorum* (Conn et al., 1994).
	B. nigra (abiotic — $CuCl_2$, Rouxel et al., 1991).	

(continued on next page)

Table 1 (*continued*)

Phytoalexin (#) (reference for first isolation)	Plant species (inducing agent, reference)	Antimicrobial activity (reference)
	B. oleracea (*P. cichorii*, Monde et al., 1990a).	
	B. rapa (abiotic — UV light, *E. carotovora, P. cichorii*, Takasugi et al., 1986, 1988; Monde et al., 1994b).	*R. solani, P. lingam* (Pedras & Okanga, 1999).
Cyclobrassininsulfoxide (**9**) (Devys, Barbier, Kollmann, Rouxel & Bousquet, 1990).	*B. carinata* (*P. lingam*, Storck & Sacristan, 1995).	*Cladosporium* sp. (Devys et al., 1990)
	B. juncea (*P. lingam*, Storck & Sacristan, 1995; Devys et al., 1990).	
	B. napus (*P. lingam*, Storck & Sacristan, 1995).	
	B. nigra (abiotic — $CuCl_2$, Rouxel et al., 1991).	
	B. oleracea (abiotic — $CuCl_2$, Rouxel et al., 1991).	
	B. rapa (abiotic — $CuCl_2$, Rouxel et al., 1991).	
	S. arvensis (*P. lingam*, Storck & Sacristan, 1995).	
Cyclobrassinone (**10**) (Gross et al., 1994).	*B. oleracea* (abiotic — UV light, Gross et al., 1994).	*C. cucumerinum* (Gross et al., 1994).
Dehydro-4-methoxy cyclobrassinin (**11**) (Monde et al., 1994b).	*B. rapa* (*P. cichorii*, Monde et al., 1994b).	*B. leersiae* (Monde et al., 1994b).
Dioxibrassinin (**16**) (Monde et al., 1991a).	*B. oleracea* (*P. cichorii*, Monde et al., 1991a).	*B. leersiae* (Monde et al., 1991a).
		P. lingam (Pedras & Okanga, 1999).
1-Methoxybrassenin A (**6**) (Monde et al., 1991b).	*B. oleracea* (*P. cichorii*, Monde et al., 1991b).	*B. leersiae* (Monde et al., 1991b).
1-Methoxybrassenin B (**7**) (Monde et al., 1991b).	*B. oleracea* (*P. cichorii*, Monde et al., 1991b).	*B. leersiae* (Monde et al., 1991b).
1-Methoxybrassinin (**3**) (Takasugi et al., 1986).	*B. carinata* (abiotic — $CuCl_2$, Rouxel et al., 1991).	*B. leersiae, P. orizae* (Takasugi et al., 1986, 1988).
	B. napus (*P. lingam*, Storck & Sacristan, 1995; Dahyia & Rimmer, 1988)	
	B. oleracea (abiotic — UV light, *P. cichorii*, Gross et al., 1994; Monde et al., 1990b).	*A. brassicae, Botrytis cinerea, C. cucumerinum, F. nivale, P. lingam, Pythium ultimum, R. solani, S. sclerotiorum,* (Dahyia & Rimmer, 1988)
	B. rapa (abiotic — UV light, *E. carotovora, P. cichorii*, Takasugi et al., 1986, 1988; Monde et al., 1994b).	*C. cucumerinum* (Gross et al., 1994)
	R. sativus (*P. cichorii*, Monde et al., 1995).	
4-Methoxybrassinin (**4**) (Monde et al., 1990b).	*B. oleracea* (*P. cichorii*, Monde et al., 1990b).	*B. leersiae* (Monde et al., 1990b, 1994b).
	B. rapa (*P. cichorii*, Monde et al., 1994b).	
1-Methoxybrassitin (**5**) (Takasugi et al., 1988).	*B. oleracea* (abiotic — UV light, *P. cichorii*, Gross et al., 1994; Monde et al., 1990b).	*C. cucumerinum* (Gross et al., 1994).
	B. rapa (*P. cichorii*, Takasugi et al., 1988).	*B. leersiae, P. orizae* (Takasugi et al., 1988; Monde et al., 1990b, Monde et al., 1995).
	R. sativus (*P. cichorii*, Monde et al., 1990b, 1995).	
6-Methoxycamalexin (**24**) (Browne et al., 1991).	*C. bursa-pastoris* (*A. brassicae*, Jimenez et al., 1997).	*A. brassicae, Cladosporium* sp. (Browne et al., 1991; Jimenez et al., 1997).
	C. sativa (*A. brassicae*, Browne et al., 1991).	
1-Mehoxyspirobrassinin (**13**) (Gross et al., 1994)	*B. oleracea* (abiotic - UV light, Gross et al., 1994).	*C. cucumerinum* (Gross et al., 1994).
1-Methoxyspirobrassinol (**14**) (Monde et al., 1995).	*R. sativus* (*P. cichorii*, Monde et al., 1995).	*B. leersiae* (Monde et al., 1995).
1-Methoxyspirobrassinol methylether (**15**) (Monde et al., 1995).	*R. sativus* (*P. cichorii*, Monde et al., 1995).	*B. leersiae* (Monde et al., 1995).
1-Methylcamalexin (**25**) (Jimenez et al., 1997).	*C. bursa-pastoris* (*A. brassicae*, Jimenez et al., 1997).	*Cladosporium* sp. (Jimenez et al., 1997)

Table 1 (*continued*)

Phytoalexin (#) (reference for first isolation)	Plant species (inducing agent, reference)	Antimicrobial activity (reference)
Methyl 1-methoxyindole-3-carboxylate (**17**) (Pedras & Sorensen, 1998)	*Eutrewma wasabiae*, Pedras & Sorensen, 1998).	*P. lingam, P. wasabiae* (Pedras & Sorensen, 1998).
Sinalexin (**19**) (Pedras & Smith, 1998).	*Sinapis alba* (abiotic — $CuCl_2$, *A. brassica*, Pedras & Smith, 1998).	*C. cucumericum* (Pedras & Smith, 1998).
Spirobrassinin (**12**) (Takasugi, Monde, Katsui & Shirata, 1987).	*B. carinata* (*P. lingam*, Storck & Sacristan, 1995).	*C. cucumerinum* (Storck & Sacristan, 1995).
	B. juncea (*P. lingam*, Storck & Sacristan, 1995).	*C. cucumerinum* (Storck & Sacristan, 1995).
	B. napus (*P. lingam*, Storck & Sacristan, 1995; Pedras & Séguin-Swartz, 1992).	*P. oryzae* (Takasugi et al., 1987).
	B. oleracea (abiotic — UV light, *P. cichorii*, Monde et al., 1990a; Gross et al., 1994).	*P. lingam* (Pedras et al., 1997b).
	B. rapa (abiotic — UV light, *P. cichorii*, Monde et al., 1990a; Monde et al., 1994b).	
	R. sativus (*P. cichorii*, Takasugi et al., 1987; Monde et al., 1995).	

1-methylindole-3-carboxylate is a constitutive antifungal metabolite isolated from the crucifer *Camelina sativa* (Jimenez, Ayer & Tewari, 1997). Despite their related biogenetic origin, the cruciferous phytoalexins have rather different structures, which would suggest substantially different biological activities; however, few correlations have been established among the different structural types and corresponding activities. In most of the cases shown in Table 1, the antimicrobial activity of phytoalexins **1**–**25** was established at the time of isolation and no further biological evaluations were made.

The antifungal activity of the phytoalexins brassinin (**1**), cyclobrassinin (**8**), spirobrassinin (**12**), brassilexin (**18**), camalexin (**23**), and dioxibrassinin (**16**) to isolates of the virulent and avirulent groups of *Phoma lingam* (Tode ex Fr.) Desm. [asexual stage of *Leptosphaeria maculans* (Desm.) Ces. et de Not.] employing a fungal radial growth assay was compared (Pedras, 1998). Brassinin and cyclobrassinin at 5×10^{-4} M completely inhibited the spore germination of both avirulent and virulent type isolates of *P. lingam* up to four days (germination of controls in 30–36 h), while brassilexin at similar concentration caused complete germination inhibition for at least two weeks. In addition, cyclobrassinin (**8**), dioxibrassinin (**16**), spirobrassinin (**12**), brassicanal A (**20**), and camalexin (**23**) did not significantly affect the mycelial growth of both avirulent and virulent type isolates of *P. lingam*, while brassinin (**1**) and brassilexin (**18**) significantly decreased the mycelial growth rate relative to control cultures. These results indicated that brassilexin (**18**) and brassinin (**1**) displayed the strongest antifungal activity against *P. lingam*. Interestingly, methoxyindole **17** was as inhibitory to virulent *P. lingam* as to *P. wasabiae* at 5×10^{-4} M, but significantly less inhibitory to avirulent *P. lingam* (Pedras & Sorensen, 1998). The fungus *Bipolaris leersiae* appeared more sensitive to brassinin (**1**) than *Phoma* species, as its growth was completely inhibited at a concentration five times lower (Kutschy et al., 1998). Camalexin (**23**), however, inhibited strongly the mycelial growth of the fungal pathogen *Rhizoctonia solani* (Pedras, 1998).

Not surprisingly, brassinin (**1**) and cyclobrassinin (**8**) showed also phytotoxic activity; leaf uptake experiments indicated that both brassinin and cyclobrassinin at 4×10^{-4} M caused substantial wilting and yellowing of petiole and foliar tissue in *B. carinata* (Pedras, Loukaci & Okanga, 1998b). A few of the phytoalexins from crucifers have been shown to inhibit the growth of cultures of human cancer cells and thus may have potential use as chemopreventive agents (Mehta et al., 1995).

3. Detection, isolation, and HPLC analysis

The detection of phytoalexins in extracts of elicited tissues of crucifers has been carried out by TLC with biodetection utilizing spores of *Cladosporium* or *Bipolaris* species, and HPLC with UV or photodiode array detection (Pedras & Sorensen, 1998). In general, following phytoalexin extraction with polar solvents like methanol, ethanol, or acetone, the detection of cruciferous phytoalexins utilizing HPLC requires a preliminary extract clean up utilizing RP-18 cartridges. Due to their polarity range, HPLC analysis of cruciferous phytoalexins has been carried out utilizing reversed phase silica gel and gradient elutions (Pedras, 1998). A good HPLC separation of thirteen phytoalexins was

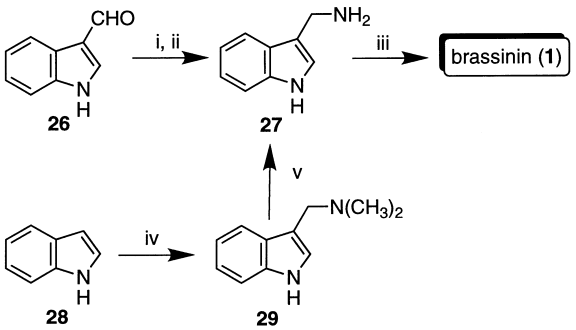
obtained utilizing an RP-18 column, ternary solvent system, and gradient elution (Monde & Takasugi, 1992), although a simpler binary system has also afforded reasonable separations (retention times obtained and conditions as shown in Table 2) (Pedras, 1998).

Similar to other phytoalexins (Ingham, 1982), the isolation of most of the cruciferous phytoalexins has been carried out utilizing multiple chromatographic steps and a TLC bioassay to detect bioactive constituents. However, because wasabi plants contained several constitutive antifungal compounds which masked the detection of phytoalexins, the isolation of methyl 1-methoxyindole-3-carboxylate (**17**) was guided by HPLC analysis (Pedras & Sorensen, 1998).

4. Synthesis

Relatively large amounts of phytoalexins are required to study their biological activity, biotransformation by phytopathogenic fungi and biosynthesis. Sufficient quantities for such studies are obtainable through synthesis, as isolation from plants is difficult and time consuming. Twenty-five phytoalexins have been reported from cruciferous plants and synthetic methods are available for 16 of them.

Brassinin (**1**) was synthesized from indole-3-carboxaldehyde (**26**) as shown in Scheme 1 (Takasugi, Monde, Katsui & Shirata, 1988). The aldehyde **26** was allowed to react with hydroxylamine hydrochloride to give a mixture of (E)- and (Z)-oximes, which after reduction with Devarda's alloy yielded indole-3-methanamine (**27**). Reaction of amine **27** with carbon disulfide in the presence of pyridine and triethylamine gave a dithiocarbamate salt, which was subsequently methylated with methyl iodide to give brassinin (**1**). Modifications of this procedure employing shorter reaction time (Pedras, Borgmann & Taylor, 1992) or a different catalyst (Mehta et al., 1995) and resulting in a higher yields of brassinin was also reported. By



Scheme 1. Reagents: (i) NH_2OH:HCL; (ii) Devarda's alloy, NaOH/MeOH; (iii) py, Et_3N, CS_2; MeI; (iv) HCHO, Me_2NH, AcOH; (v) MeI; NH_4OH.

Table 2
HPLC Retention times[a] of selected cruciferous phytoalexins (Pedras, 1998)

Phytoalexins	Retention time[b] (min)
Brassicanal A (**20**)	10.2
Brassilexin (**18**)	11.7
Brassinin (**1**)	18.7
Brassinin homolog (**79**)	20.3
Camalexin (**23**)	28–30
Cyclobrassinin (**8**)	25.5
Cyclobrassininsulfoxide (**9**)	12.3
Dioxibrassinin (**16**)	7.5
1-Methoxybrassinin (**3**)	24.0
1-Methoxybrassenin B (**7**)	23.3
Methyl 1-methoxyindole-3-carboxylate (**17**)	18.4
Sinalexin (**19**)	19.3
Spirobrassinin (**12**)	12.1

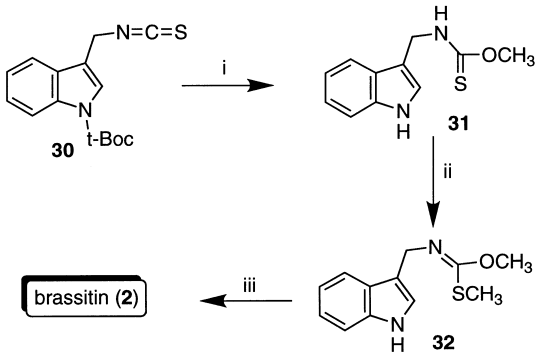
[a] Analyses performed with a liquid chromatograph equipped with quaternary pump, automatic injector, photodiode array detector, degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 i.d. × 200 mm), equipped with an in-line filter; mobile phase 75% H_2O–25% CH_3CN to 100% CH_3CN, for 35 min, linear gradient, and a flow rate = 1.0 ml/min.

[b] Retention times are within ±0.2 min, except for camalexin which has a concentration dependent retention time under these conditions.

using trideuterated methyl iodide in the final step, deuterated brassinin was obtained for use in biosynthetic studies (Monde, Takasugi & Ohnishi, 1994a). In another three step procedure brassinin (**1**) was synthesized from indole (**28**) in 58% overall yield (Yamada, Kobayashi, Shimizu, Aoki & Somei, 1993). In this process indole was converted to gramine (**29**) and the latter methylated with methyl iodide to the quaternary salt. Nucleophilic displacement with concentrated ammonia solution yielded indole-3-methanamine (**27**). Subsequent reaction with carbon disulfide and methyl iodide yielded brassinin (**1**).

Brassinin (**1**) was also synthesized by employing isothiocyanate **30** as a putative biomimetic intermediate (Kutschy et al., 1998). In this synthesis indole-3-carboxaldehyde (**26**) was allowed to react with $(t\text{-Boc})_2O$ to yield the *N*-protected aldehyde which, after reaction with hydroxylamine hydrochloride, provided a mixture of oximes. After reduction of the oxime mixture, the resulting amine treated with thiophosgene to yield isothiocyanate **30**. Nucleophilic addition of sodium methanethiolate to isothiocyanate **30** yielded the *t*-Boc protected brassinin. Deprotection with sodium methanethiolate in the presence of 15-crown-5-ether and piperidine gave brassinin (**1**).

Starting from isothiocyanate **30**, brassitin (**2**) was synthesized in three steps (see Scheme 2) (Kutschy et al., 1998). Treatment of **30** with sodium methoxide gave compound **31** which was methylated and hydrolyzed under acidic conditions to afford brassitin (**2**) in 39% yield. Brassitin (**2**) was also prepared by oxi-



Scheme 2. Reagents: (i) MeONa, MeOH; (ii) MeONa, MeOH: MeI; (iii) HCl, THF.

dation of brassinin with hydrogen peroxide in the presence of *p*-toluenesulfonic acid and triphenylphosphine (Monde, Takasugi & Shirata, 1995). This process afforded the phytoalexin in 8% yield.

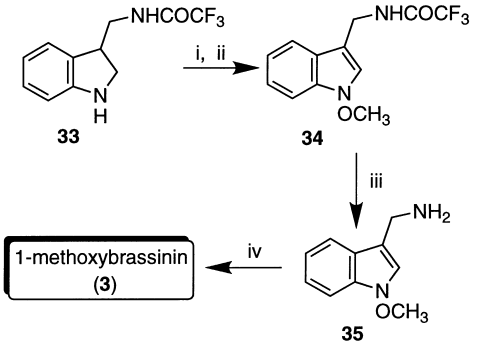
1-Methoxybrassinin (**3**) was synthesized in seven steps from indole (**28**) with an overall yield of 22% (Scheme 3) (Somei, Kobayashi, Shimizu & Kawasaki, 1992). Thus, indole was converted to gramine (**29**) which yielded indole-3-methanamine (**27**) upon treatment with methyl iodide and concentrated ammonia solution. Indole-3-methanamine (**27**) was then converted to its trifluoroacetyl derivative by treatment with ethyl trifluoroacetate. Reduction with triethylsilane in trifluoroacetic acid afforded the corresponding indoline **33** which upon catalytic oxidation with sodium tungstate and hydrogen peroxide, followed by methylation with diazomethane produced **34** in 77% yield. Subsequent alkaline hydrolysis in methanol–water produced 3-aminomethyl-1-methoxyindole (**35**) in 98% yield. The latter compound was readily converted to 1-methoxybrassinin (**3**) by treatment with carbon disulfide and methyl iodide in 64% yield.

4-Methoxybrassinin (**4**) was synthesized from indole-3-carboxaldehyde in four steps (see Scheme 4) (Yamada et al., 1993). After conversion of indole-3-carboxaldehyde (**26**) to 4- methoxyindole-3-carboxaldehyde (**36**) followed by reductive amination to give 3-dimethylaminomethyl-4-methoxyindole (**37**), reaction with sodium borohydride in ammonium hydroxide produced 4-methoxyindole-3-methanamine (**38**). 4-Methoxybrassinin (**4**) was obtained in 64% yield upon treatment of **38** with carbon disulfide and methyl iodide. 1-Methoxybrassenin A (**6**) was obtained by methylation of 1-methoxybrassinin (**3**) with methyl iodide (Monde & Takasugi, 1991).

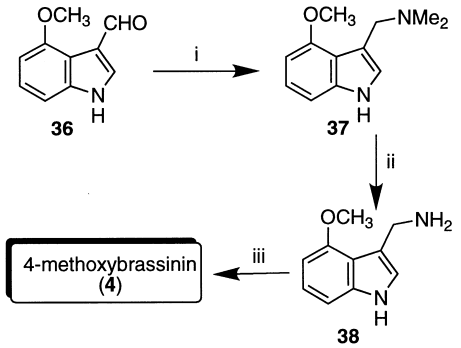
Cyclobrassinin (**8**) was synthesized by cyclization of brassinin (**1**). Bromination of brassinin (**1**) with pyridinium bromide perbromide in dichloromethane followed by dehydrobromination with DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) and subsequent column chromatography provided cyclobrassinin (**8**) in 35% yield (Takasugi et al., 1988). The cyclization has also been accomplished with NBS (*N*-bromosuccinimide) and triethylamine in place of pyridinium bromide perbromide and DBU (Mehta et al., 1995). [Methyl-$^2H_3$]cyclobrassinin for use in biosynthetic studies was obtained in 25% yield from [methyl- $^2H_3$]brassinin by cyclization with pyridinium bromide perbromide and DBU (Monde et al., 1994a). In another procedure cyclobrassinin (**8**) was synthesized from *N*-protected brassinin prepared from isothiocyanate **30** (Kutschy et al., 1998). The phytoalexin cyclobrassinin sulfoxide (**9**) was prepared from cyclobrassinin (**8**) upon oxidation with *m*-chloroperoxybenzoic acid to give the phytoalexin cyclobrassinin sulfoxide in 80% yield (Devys & Barbier, 1992).

Dehydro-4-methoxycyclobrassinin (**11**) was obtained in two steps from 4-methoxybrassinin (**4**) (Monde et al., 1994b). Treatment of 4-methoxybrassinin with NBS followed by dehydrobromination with DBU afforded 4-methoxycyclobrassinin which was oxidized with DDQ (2,3-dichloro-5,6-dicyano-1,4-benzoquinone) to yield dehydro-4-methoxycyclobrassinin (**11**) in 34% yield.

(±)-Dioxibrassinin (**16**) was synthesized from 3-(aminomethyl)-3-hydroxyindole (**39**) by treatment with car-

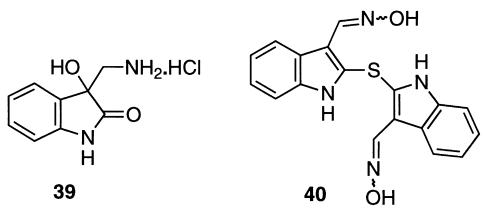


Scheme 3. Reagents: (i) $Na_2WO_4{\cdot}2H_2O$, H_2O_2; (ii) CH_2N_2, Et_2O; (iii) NaOH; (iv) py, Et_3N, CS_2; MeI.



Scheme 4. Reagents: (i) $NaBH_4$, Me_2NH; (ii) $NaBH_4$, NH_4OH; (iii) py, Et_3N, CS_2; MeI.

bon disulfide in the presence of triethylamine and pyridine followed by methylation with methyl iodide (Monde, Sasaki, Shirata & Takasugi, 1991a). This process gave dioxibrassinin (**16**) in 75% yield. By employing trideuterated methyl iodide for the methylation step (±)-[methyl-$^2H_3$]dioxibrassinin was obtained for use in biosynthetic studies (Monde et al., 1994a). Spirobrassinin (**12**) was synthesized by treatment of dioxibrassinin with thionyl chloride (Monde et al., 1994a).



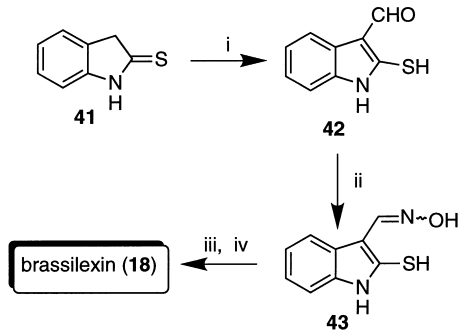
Brassilexin (**18**) was synthesized in 18% overall yield in a three step procedure starting with indole-3-carboxaldehyde (Devys & Barbier, 1990b). The aldehyde was treated with hydroxylamine hydrochloride to yield a mixture of (E)- and (Z)-oximes, which upon reaction with sulfur chloride in acetic acid provided the monosulfide **40** in 67% yield. The monosulfide cyclized to brassilexin (**18**) at room temperature upon treatment with polyphosphoric acid. In a one-pot procedure also starting with indole-3-carboxaldehyde, brassilexin was synthesized in 30% yield (Devys & Barbier, 1993). The aldehyde was first treated with sulfur monochloride in acetic acid to yield a disulfide which, after removal of excess acid, was treated with ammonia in methanol to provide brassilexin. Brassilexin (**18**) was also synthesized by oxidative ring contraction of cyclobrassinin (**8**) with *m*-chloroperoxybenzoic acid to give cyclobrassinin sulfoxide (**9**). The latter compound was then oxidized with sodium periodate to provide brassilexin (**18**) in 60% yield from cyclobrassinin sulfoxide (Devys & Barbier, 1992). Direct oxidation of cyclobrassinin with sodium periodate gave brassilexin in a lower yield (30%) (Devys & Barbier, 1990a).

In an efficient four step procedure brassilexin (**18**) was synthesized from 2-indolinethione (**41**) in 64% overall yield (Scheme 5) (Pedras & Okanga, 1998b). Formylation of 2-indolinethione (**41**) with ethyl formate gave 2-mercaptoindole-3-carboxaldehyde (**42**) in 92% yield. Treatment of this aldehyde with hydroxylamine hydrochloride under standard conditions quantitatively yielded a mixture of (E)- and (Z)-oximes (**43**). Reduction of the oxime mixture with sodium cyanoborohydride in the presence of titanium chloride yielded 3- methylenaminoindole-2-thione which upon treatment with activated charcoal in methanol afforded brassilexin (**18**).

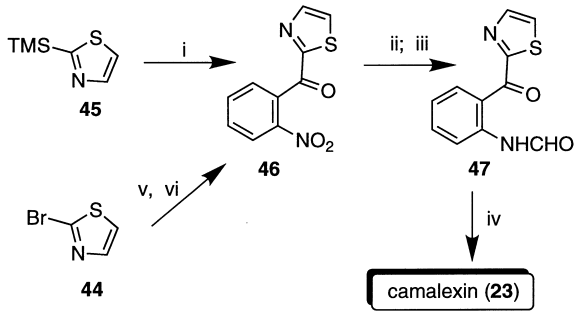
Brassicanal A (**20**) was synthesized by Vilsmeier formylation of 2-(methylthio) indole (Monde, Katsui, Shirata & Takasugi, 1990a). In another synthesis, brassicanal A (**20**) was obtained by methylation of 2-mercaptoindole-3-carboxaldehyde (**42**) with diazomethane (Pedras & Okanga, 1998b). Brassicanal B (**21**) was synthesized by treatment of 2-indolinethione (**41**) with bromoacetone followed by Vilsmeier formylation (Monde et al., 1990b).

Camalexin (**23**) was synthesized from indole in a two step procedure (Ayer, Craw, Ma & Miao, 1992). Reaction of indolylmagnesium iodide (prepared in situ from indole and methylmagnesium iodide) with 2-bromothiazole (**44**) in refluxing benzene afforded camalexin (**23**) in 68–76% yield. In a four step procedure camalexin was synthesized from 2-trimethylsilyl thiazole (**45**) (Scheme 6) (Fürstner & Ernst, 1995). The latter compound provided ketone **46** in good yield upon acylation with 2-nitrobenzoyl chloride. Standard hydrogenation of **46** over palladium on charcoal followed by formylation of the resulting amino group with formic acid and acetic anhydride gave oxoamide **47**, which was reductively cyclized upon heating with titanium chloride and zinc dust in DME (1,2-dimethoxyethane) to afford camalexin (**23**) in 71% yield. Camalexin (**23**) was also synthesized from 2-bromothiazole (**44**) by an alternative procedure amenable to large scale preparations (Scheme 6) (Fürstner & Ernst, 1995). Lithiation of 2-bromothiazole (**44**) and subsequent reaction with 2-nitrobenzaldehyde gave an alcohol, which was oxidized to the corresponding ketone **46** using pyridinium dichromate (PDC) in dichloromethane.

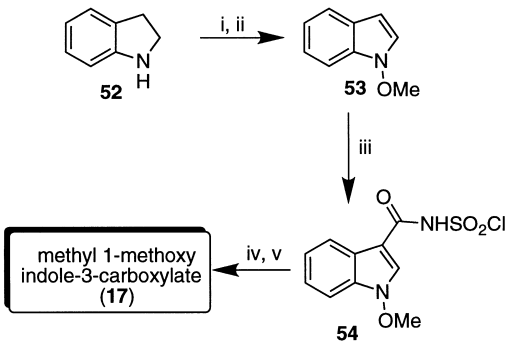
6-Methoxycamalexin (**24**) was obtained from 4-methyl-3-nitrophenol, prepared in 75% overall yield from 4-methyl-3-nitroaniline (**48**), and then methylated to yield nitroanisole 49 (Scheme 7) (Ayer et al., 1992). Treatment of 4-methyl-3-nitroanisole (**49**) with dimethylformamide dimethyl acetal and pyrrolidine gave β-pyrrolidinostyrene (**50**) which was reduced with titanium chloride in ammonium acetate buffer to



Scheme 5. Reagents: (i) NaH, HCOOEt; (ii) $NH_2OH_4 \cdot HCl$, NaOAc, EtOH; (iii) $NaBH_3CN$, $TiCl_3$, MeOH; (iv) activated charcoal.



Scheme 6. Reagents: (i) 2-nitrobenzoyl chloride, CH_2Cl_2; (ii) H_2, Pd-charcoal, EtOAc; (iii) HCOOH, Ac_2O; (iv) $TiCl_3$, Zn-dust, DME, reflux; EDTA disodium salt, H_2O; (v) *n*-BuLi, Et_2O, −78°C; 2-nitrobenzaldehyde, Et_2O, −78°C; (vi) PDC, CH_2Cl_2.



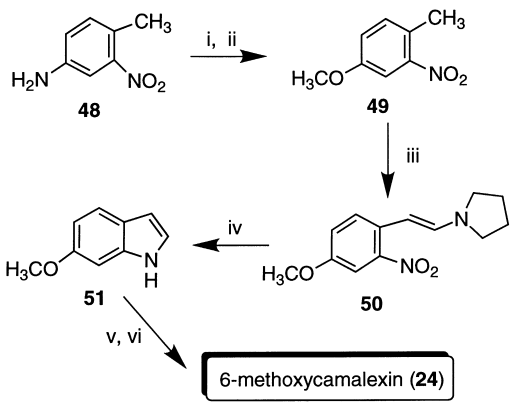
Scheme 8. Reagents: (i) $Na_2WO_4{\cdot}2H_2O$, H_2O_2; (ii) CH_2N_2, Et_2O; (iii) $ClSO_2NCO$; (iv) NaOH; (v) CH_2N_2, Et_2O.

afford 6-methoxyindole (**51**) in 73–78% overall yield. Alkylation of 6-methoxyindolyl magnesium iodide with 2-bromothiazole (**44**), according to the procedure used to prepare camalexin, gave 6-methoxycamalexin (**24**) in good yield.

The phytoalexin methyl 1-methoxyindole-3-carboxylate (**17**) was obtained from indoline (**52**) in six steps (Scheme 8), requiring only two chromatographic separations (Pedras & Sorensen, 1998), a simplification of a previously published procedure (Acheson, Aldridge, Choi, Nwankwo, Ruscoe & Wallis, 1984). Indoline was reacted with sodium tungstate and hydrogen peroxide, followed by treatment with diazomethane to yield 1-methoxyindole (**53**). Chlorosulfonylisocyanate was added to methoxyindole to yield **54** which upon alkaline hydrolysis and methylation with diazomethane yielded methyl 1-methoxyindole-3-carboxylate (**17**) in 9% overall yield.

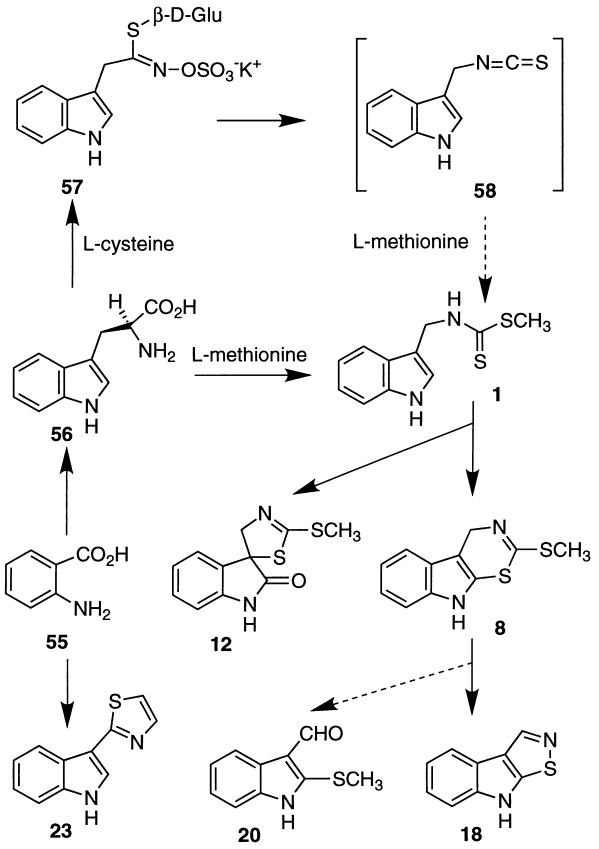
5. Biosynthesis

Scheme 9 summarizes the presently known biosynthetic relationships among the diverse cruciferous phytoalexins and their precursors. The amino acid L-tryptophan (**56**) is the biogenetic precursor of most of these phytoalexins (Monde et al., 1994a). Tryptophan is biosynthesized from anthranilic acid (**55**) via the shikimate pathway, which has been the subject of several reviews (Dewick, 1998). However, anthranilic acid (**55**), but not tryptophan is a precursor of the phytoalexin camalexin (**23**) (Tsuji, Zook, Somerville, Last & Hammerschmidt, 1993). Aside from tryptophan (**56**), closer precursors of brassinin (**1**) such as indole glucosinolate (glucobrassicin) (**57**) and indole isothiocyanate **58** were suggested (Monde et al., 1994a). In fact, indole glucosinolates such as glucobrassicin (**57**) are also biosynthesized from L-tryptophan and are known to give the respective isothiocyanates upon enzymic hydrolysis followed by a Lossen-type rearrangement (Fenwick et al., 1989). Furthermore, time-course studies in UV-irradiated turnip root tissue (*B. campestris* L. ssp. *rapa*) indicated that the levels of indole glucosinolates and phytoalexins increased in the UV-irradiated tissue, whereas only indole glucosinolate levels increased in non-irradiated control tissue (Monde, Takasugi, Lewis & Fenwick, 1991). As depicted in Scheme 9, it is likely that brassinin (**1**) derives via isothiocyanate **58** from glucobrassicin (**57**), although a related intermediate cannot be ruled out (Monde et al., 1994a).



Scheme 7. Reagents: (i) $NaNO_2$, H_2SO_4; H_2O; (ii) Me_2SO_4, K_2CO_3, MeCN; (iii) dimethylformamide dimethyl acetal, pyrrolidine; (iv) $TiCl_3$, NH_4OAc; (v) MeMgI, Et_2O; (vi) 2-bromothiazole, benzene.

The biosynthesis of brassinin (**1**), cyclobrassinin (**8**) and spirobrassinin (**12**) was studied using isotopically labeled (^{13}C and ^{2}H) precursors administered to turnip tissue obtained from turnip root (*B. campestris* L. ssp. *rapa*) (Monde et al., 1994a; Monde & Takasugi, 1991). In order to confirm the origin of the indole moiety of brassinin, incorporation of L-tryptophan was examined. When L-[4′-^{2}H]tryptophan was administered to UV-irradiated turnip tissue and incubated for 37 h, spirobrassinin (**12**) was isolated as the main metabolite. Spectroscopic analysis revealed incorporation of the ^{2}H label into the oxindole nucleus of both **8** and **12**. A feeding experiment with L-[methyl-$^{2}H_3$]methionine indi-



Scheme 9.

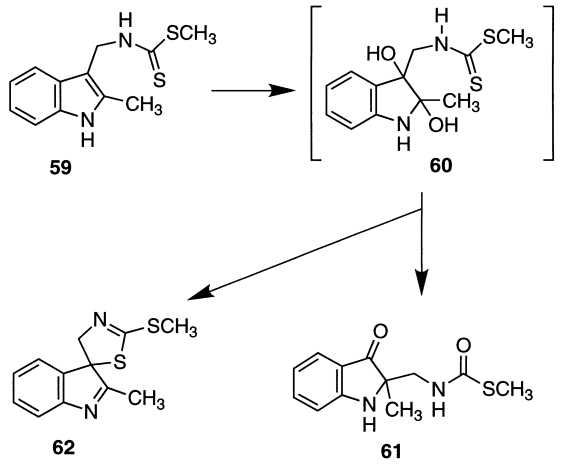
cated that the methyl groups of brassinin, cyclobrassinin, and spirobrassinin derived from L-methionine. Administration of [methyl-$^{2}H_3$]brassinin to the turnip tissue, followed by incubation led to effective incorporation of the ^{2}H label into cyclobrassinin (**8**) and spirobrassinin (**12**). These results demonstrated that L-tryptophan (**56**) was the precursor of brassinin (**1**) and that **1** was an advanced precursor of cyclobrassinin (**8**) and spirobrassinin (**12**). However, neither cyclobrassinin (**8**) nor dioxibrassinin (**16**) were incorporated into spirobrassinin (**12**) (Monde et al., 1994a). In addition, it was shown that the thiocarbonyl carbon of brassinin (**1**), and the imino carbon of spirobrassinin (**12**), originated from the C-2 carbon of tryptophan (Monde & Takasugi, 1991). An analogous experiment with DL-[2-^{13}C]tryptophan resulted in a four-fold enhancement of the imino carbon NMR signal of spirobrassinin and indicated the involvement of a molecular rearrangement in the pathway from tryptophan to brassinin. This result was suggestive of the isothiocyanate **58** as a key intermediate to brassinin (**1**).

To examine the possible role of isothiocyanates in the biosynthesis of cruciferous phytoalexins, benzyl isothiocyanate was chosen as a model substrate and administered to the turnip tissue (Monde et al., 1994a). A new metabolite was isolated and identified as methyl benzyldithiocarbamate by direct comparison with a synthetic sample. Formation of this metabolite in the turnip tissue indicated that indol-3-ylmethyl isothiocyanate (**58**) (Scheme 9) was involved in the biosynthesis of brassinin. In order to detect the labile indol-3-ylmethyl isothiocyanate intermediate, a trapping experiment with methanethiolate was done (Monde et al., 1994a). Turnip roots were homogenized with 15% aqueous sodium methanethiolate and the homogenate extracted with ethyl acetate. Fractionation of this extract gave brassinin (**1**), which was not isolated in the absence of sodium methanethiolate. The isolation of brassinin (**1**) suggested a transient formation of isothiocyanate **58** as a reaction intermediate.

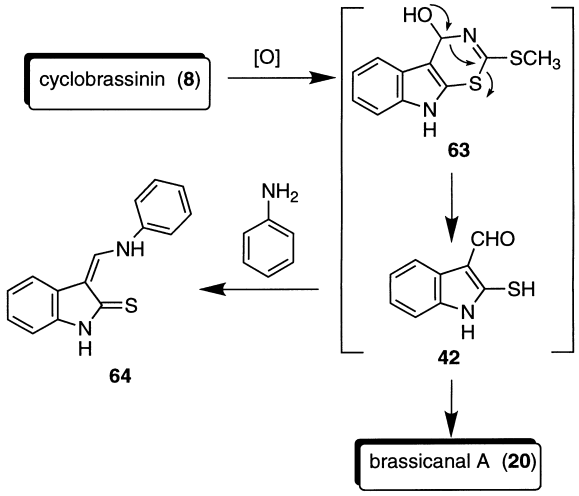
In order to clarify whether the methylthio group of brassinin (**1**) was introduced directly or in a stepwise manner, a mixture of L-[methyl-$^{3}H_3$]methionine and L-[^{35}S]methionine was simultaneously administered to UV-elicited turnip tissue (Monde et al., 1994a). Forty-eight hours after administration of the mixture, metabolites were extracted and separated to yield brassinin (**1**) and spirobrassinin (**12**). The ratio of $^{35}S/^{3}H$ of isolated brassinin and spirobrassinin was unchanged during biosynthesis of brassinin (**1**) and spirobrassinin (**12**) an indication that brassinin was biosynthesized by intact incorporation of the methylthio group from L-methionine into the isothiocyanate **58**. Further experiments with L-[^{35}S]cysteine supported the conclusion that the thiocarbonyl sulfur atom of brassinin (**1**) originated from L-cysteine.

Production of a variety of indole-related phytoalexins can be explained by postulating a common intermediate which bridges brassinin and other cruciferous phytoalexins (Monde et al., 1994a). To determine the nature of the intermediate, 2-methylbrassinin (**59**) (Scheme 10) was chosen as a probe. Since compound **59** had a methyl group at the C-2 position, the formation of a cyclobrassinin-type or a spirobrassinin-type structure was structurally prohibited, thus isolation of a corresponding intermediate was expected. After dithiocarbamate **59** was administered to UV-elicited turnip tissue and incubated, two new compounds were isolated which were not detected in the control tissue. Spectroscopic analysis led to their identification as **61** and **62**. Formation of these two metabolites (Scheme 10) suggested the diol **60** as the immediate precursor, likely via a pinacol-type rearrangement (Monde et al., 1994a).

The co-occurrence of 1-methoxybrassinin (**3**) with brassinin (**1**) and cyclobrassinin (**8**) in elicited Chinese cabbage and Japanese radish suggested that *N*-hydroxylation of brassinin, followed by biological methylation could lead directly to **3** or **8**, through eliminative cyclization (Monde et al., 1995). However, [methyl-$^{2}H_3$]brassinin was incorporated into cyclobrassinin (**8**)



Scheme 10



Scheme 11

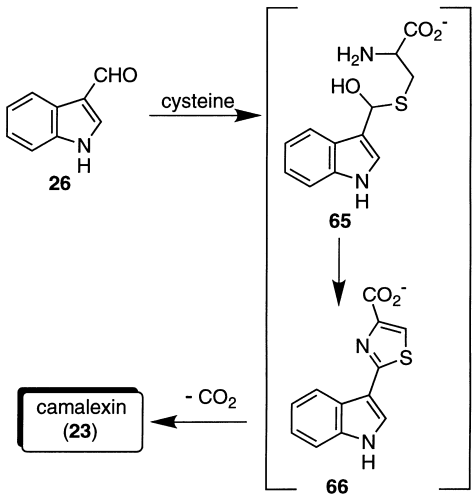
but not into 1-methoxybrassinin (**3**) which indicated that the latter is not derived from brassinin.

The biosynthesis of indole phytoalexins in kohlrabi (*B. oleracea* var. *gongylodes*) was also studied using radiolabeled precursors (Gross, Porzel & Schmidt, 1994). Administering both L-[2- ^{14}C]tryptophan and L-[$^{14}CH_3$]methionine to UV-irradiated stem tubers of kohlrabi indicated that the sulfur-containing indole phytoalexins were biosynthesized from these amino acids.

Phytoalexins that contain only one sulfur atom, such as brassicanal A (**20**) (Monde et al., 1990a) are thought to be biosynthesized from indole phytoalexins that contain two sulfur atoms. In a unique trapping of a potential biosynthetic intermediate to brassicanal A (**20**), aniline or acetanilide was added to UV-irradiated turnip tissue (Monde, Tanaka & Takasugi, 1996). The structure of the final product **64** (Scheme 11) strongly suggested that 2-mercaptoindole-3- carboxaldehyde (**42**) was a biosynthetic intermediate from brassinin (**1**) to brassicanal A (**20**). The expected precursor **42** or its thione tautomer could be formed by hydrolysis of dehydrocyclobrassinin via intermediate **63**. Although dehydrocyclobrassinin has not yet been isolated, a methoxy derivative (**11**) was isolated from elicited turnip root (Monde et al., 1994b). Co-occurrence of brassicanal A (**20**) with cyclobrassinin (**8**) in *P. cichorii*-inoculated Chinese cabbage appeared to support this pathway.

Brassinin (**1**) and cyclobrassinin (**8**) were shown to be intermediates in the biosynthesis of the cruciferous phytoalexin brassilexin (**18**) (Pedras et al., 1998b). Following feeding experiments with tetradeuterated brassinin (**1**) and cyclobrassinin (**8**), leaves of *B. carinata* were elicited with the blackleg causing fungus *P. lingam* and incubated. Spectroscopic and HPLC analyses indicated that both brassinin and cyclobrassinin were incorporated into the brassilexin (**18**).

Browne et al. in 1991 (Browne, Conn, Ayer & Tewari, 1991) first proposed a route for camalexin (**23**) biosynthesis that involved the condensation of indole-3-carboxaldehyde with cysteine followed by cyclization and decarboxylation (Scheme 12). Consistent with this proposal, anthranilate (**55**), but not tryptophan (**56**), was shown to be a biosynthetic precursor of camalexin (**23**) in leaves of *Arabidopsis thaliana* (Tsuji et al., 1993). Radiolabeled tryptophan (**56**) and anthranilate (**55**) of similar specific activities were administered to detached leaves of *A. thaliana* followed by treatment with silver nitrate. As an additional test of whether or not camalexin was biosynthesized from tryptophan, camalexin levels in three tryptophan-deficient mutants of Arabidopsis were measured after treatment with silver nitrate (Tsuji et al., 1993). The results of these experiments supported the hypothesis that anthranilate (**55**) but not tryptophan (**56**) was a direct biosynthetic



Scheme 12

precursor of camalexin (**23**). Recently, the origin of the sulfur atom in the thiazole ring of camalexin was established after incubation of leaves of *A. thaliana* with [^{35}S]cysteine and [^{35}S]methionine (Zook & Hammerschmidt, 1997). The incorporation of radioactivity into camalexin (**23**) provided strong evidence that the sulfur atom of the thiazole ring originated from cysteine. Additional feeding experiments with cysteine labeled with either ^{2}H or ^{13}C and ^{13}C supported the previous conclusion. This camalexin (**23**) biosynthetic pathway was confirmed to occur in cell cultures of *A. thaliana* where, not surprisingly, indole was also established to be a precursor of camalexin (**23**) (Zook, 1998).

6. Microbial biotransformations

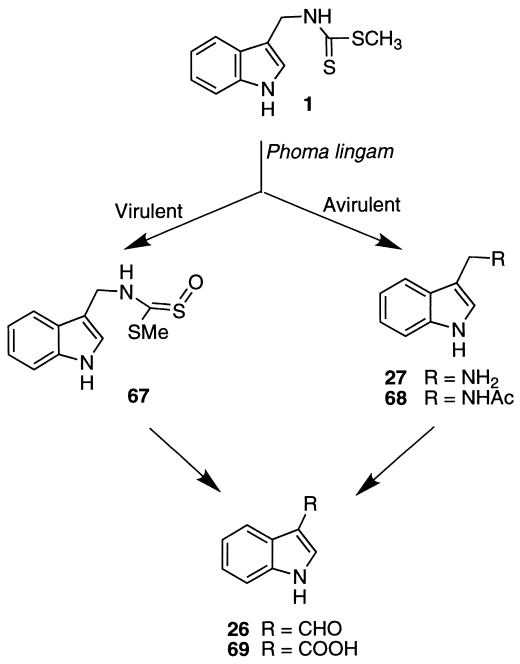
Phytopathogenic fungi are able to circumvent some of the plant chemical defences through metabolism and detoxification. When phytopathogenic fungi can effectively detoxify phytoalexins, the outcome of the plant-pathogen interaction can favor the pathogen and be detrimental to the plant. To date, several examples demonstrate that fungal pathogens can detoxify cruciferous phytoalexins efficiently (Pedras, 1998). Multiple examples of phytoalexin detoxification have been reported in other plant families (VanEtten, Mathews & Mathews, 1989; VanEtten, Sandrock, Wasmann, Soby, McCluskey & Wang, 1995; Daniel & Purkayastha, 1995).

The biotransformation of the phytoalexin brassinin by the blackleg fungus (*P. lingam*) was investigated (Pedras et al., 1992; Pedras & Taylor, 1991; Pedras & Taylor, 1993) and it was determined that its virulence correlated with its ability to rapidly metabolise and detoxify brassinin (Pedras et al., 1992). Incubation of the virulent *P. lingam* isolates with brassinin (**1**) resulted in detection and isolation of three metabolites. The unusual structure of the first metabolic intermediate was assigned as methyl (3-indolylmethyl)dithiocarbamate S-oxide (**67**) based on spectroscopic data and synthesis of a methyl derivative. The other two metabolites were readily identified as indole-3-carboxaldehyde (**26**) and indole-3-carboxylic acid (**69**) from spectroscopic data and comparison with authentic samples. Further biotransformation studies on the three metabolites by virulent *P. lingam* established the biotransformation pathway from brassinin (**1**) as shown in Scheme 13.

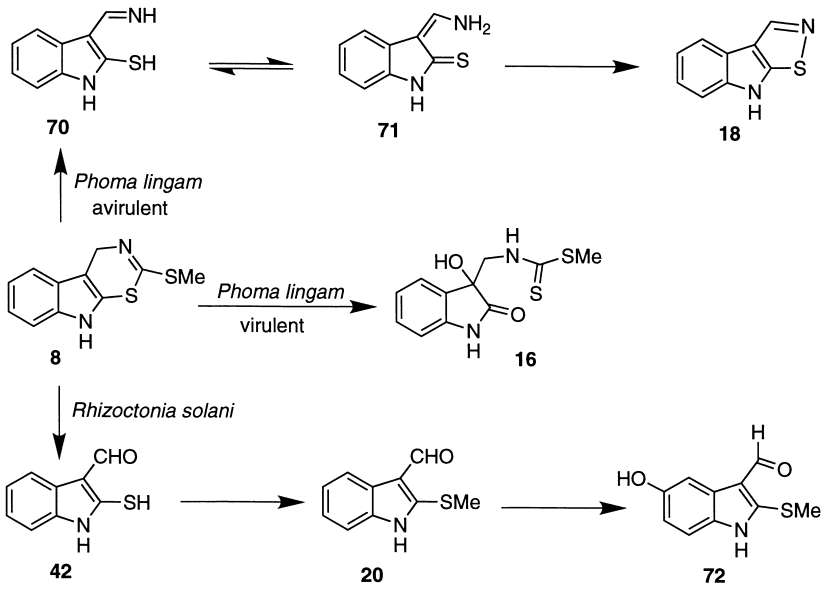
Further investigation (Pedras & Taylor, 1993) on the metabolism of brassinin by avirulent isolates of *P. lingam* indicated a metabolic pathway which is different from that of virulent isolates. The metabolism of brassinin (**1**) by avirulent isolates resulted in the detection and isolation of four metabolites: indole-3-carboxaldehyde (**26**), indole-3-carboxylic acid (**69**), indole-3-methanamine (**27**), and *N*-acetyl-3-indolyl-methylamine (**68**), as shown in Scheme 13. While in virulent isolates the transformation of brassinin (**1**) rapidly yielded aldehyde **26** via intermediate **67**, in avirulent isolates brassinin (**1**) was slowly converted to the aldehyde **26** via intermediates **27** and **68** (Pedras & Taylor, 1993).

The antifungal activity of brassinin (**1**) and its metabolites was compared using spore germination and radial mycelial growth assays (Pedras & Taylor, 1993). The results of these bioassays indicated that the biotransformation of brassinin (**1**) by virulent and avirulent isolates of *P. lingam* was a detoxification, as brassinin metabolites had no significant antifungal activity. In addition, it was established that brassinin (**1**) inhibited the biosynthesis of nonselective phytotoxins in *P. lingam*. These phytotoxins were not detected in fungal cultures incubated with brassinin (**1**) until 6–8 h after complete brassinin (**1**) transformation. By contrast, incubation with any of the intermediates **26**, **27**, **67**, **68**, and **69** did not noticeably affect phytotoxin production.

Recent work showed a striking difference between the metabolism of cyclobrassinin (**8**) by avirulent type and virulent type isolates of *P. lingam*, as shown in Scheme 14 (Pedras & Okanga, 1998a, 1999). HPLC analysis of organic extracts of cultures of avirulent isolates incubated with **8** indicated a rapid decrease in the concentration of **8** and the concurrent appearance of two additional constituents. Spectroscopic analysis established that one of the constituents was the known phytoalexin brassilexin (**18**) and the other constituent,



Scheme 13

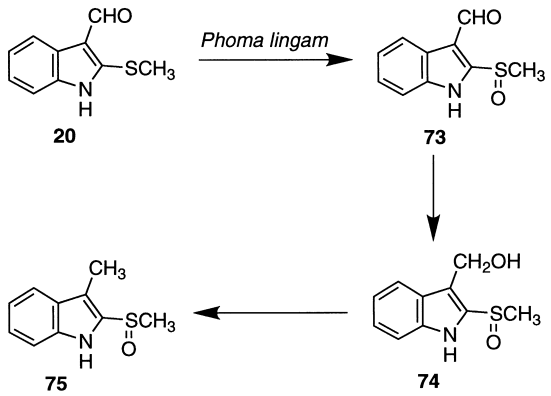


Scheme 14

a relatively less stable metabolite, a mixture of 3-methylenaminoindole-2-thione (**71**) and related tautomers (e.g. **70**). Two days after incubation of the avirulent isolate with cyclobrassinin (**8**), no brassilexin (**18**) or other phytoalexins or putative metabolites were detected in any of the cultures, or their extracts. Similar experiments carried out with virulent isolates of *P. lingam* incubated with cyclobrassinin (**8**) afforded yet another known phytoalexin, dioxibrassinin (**16**). Two days after incubation of a virulent isolate with cyclobrassinin (**8**), no phytoalexins or derivatives were detected in any of the cultures or their extracts. Additional biotransformation studies with *R. solani* established that this pathogen metabolized and detoxified cyclobrassinin (**8**) via the phytoalexin brassicanal A (**20**), which was further metabolized into nontoxic products (Scheme 14). The intermediate **42** could explain the formation of **20** through enzymatic oxidation of **8**. These remarkable results demonstrated that cyclobrassinin (**8**) was detoxified via the phytoalexins dioxibrassinin (**16**), brassilexin (**18**), or brassicanal A (**20**), depending on the fungal species, as shown in Scheme 14 (Pedras & Okanga, 1999). It is worth noting that cyclobrassinin (**8**) was demonstrated to be an in planta biosynthetic precursor of brassilexin (**18**) (Pedras et al., 1998b) and also proposed to be a biosynthetic precursor of brassicanal A (**2**) (Monde et al., 1996). Therefore, it was suggested that some phytopathogens may have acquired a more effective mechanism for overcoming this chemical defense by adopting biosynthetic pathways operating in planta. This strategy appeared quite plausible, especially considering that most fungal pathogens have coevolved with plants for innumerable generations (Pedras & Okanga, 1999).

The biotransformation of the phytoalexin brassicanal A (**20**) by *P. lingam* led to the detection and isolation of three metabolites: brassicanal A sulfoxide (**73**), 3-hyroxymethylindole-2-methylsulfoxide (**74**), and 3-methylindole-2-methylsulfoxide (**75**), as shown in Scheme 15 (Pedras & Khan, 1996; Pedras, Khan & Taylor, 1997b). In the first biotransformation step, the SCH_3 group of brassicanal A was oxidized to the corresponding sulfoxide **73**. In subsequent steps the aldehyde group of **20** was reduced to the alcohol **74** and then further to the 3-methylindole **75**. The biotransformation of brassicanal (**20**) A was shown to be a detoxification, since the antifungal activities of brassicanal A (**20**) and its biotransformation products indicated that brassicanal A (**20**) was significantly more inhibitory to *P. lingam* than the products **73**–**75**.

Studies on the biotransformation of the phytoalexin brassilexin (**18**) and dioxibrassinin (**16**) by *P. lingam*



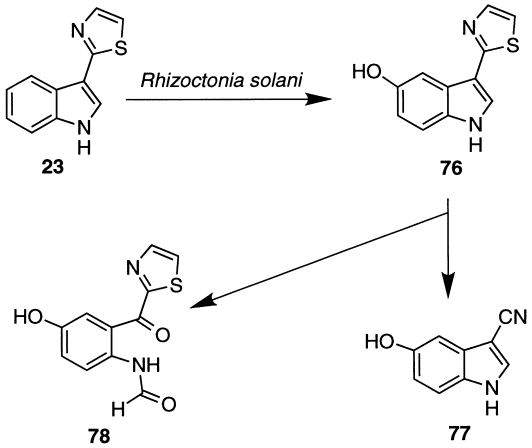
Scheme 15

indicated that the phytoalexins were transformed but no biotransformation products were detected or isolated (Pedras, 1998; Pedras et al., 1997b). This work confirmed the results of a previous study on fungal metabolism of brassilexin (**18**) (Rouxel, Kollman & Ballesdent, 1995). Possible metabolic products of brassilexin might be very polar and more soluble in the aqueous medium than in organic solvents, thus precluding extraction and detection. On the other hand, spirobrassinin (**12**) was stable in fungal cultures and did not appear to be metabolized or interfere with the production of fungal toxins (Pedras, 1998).

Although blackleg fungi could metabolize and detoxify brassinin (**1**), cyclobrassinin (**8**), dioxibrassinin (**16**), brassilexin (**18**), and brassicanal A (**20**), camalexin (**23**) was not metabolized by either virulent or avirulent type isolates of *P. lingam*. None of the blackleg fungal isolates tested metabolized camalexin (**23**) or appeared to be affected by a concentration of 5×10^{-4} M (Pedras et al., 1997b; Pedras, Khan & Taylor, 1998a). Camalexin (**23**) was, however, metabolized by *Rhizoctonia solani*, a pathogen of *Camelina sativa* (false flax), a plant that produces camalexin (**23**) (Pedras & Khan, 1997). Incubation of *R. solani* with camalexin (**23**) resulted in the detection and isolation of metabolites **76**, **77**, and **78**, whose structures were deduced from their respective spectroscopic data and confirmed by synthesis. Further biotransformation of these metabolites led to establishment of the biotransformation sequence shown in Scheme 16. This was the first time that oxidation of the indole ring of a cruciferous phytoalexin was observed. Previous work with other cruciferous phytoalexins indicated that fungal oxidation occurred at the indole substituents at C-2 or C-3.

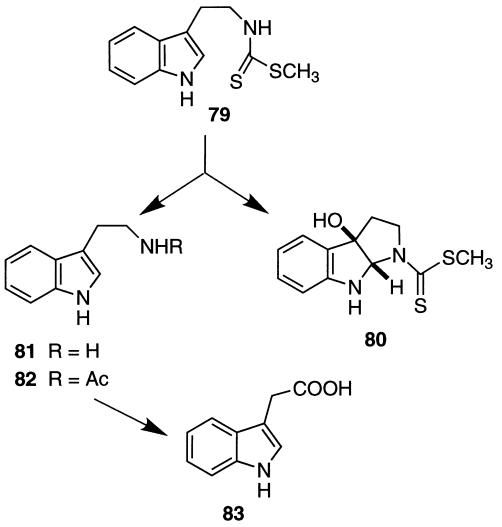
The biotransformation products of camalexin (**23**) by *R. solani* were found to be significantly less toxic than camalexin (**23**) itself. While agar plates containing camalexin (**23**) at 5×10^{-4} M inoculated with *R. solani* showed no mycelial growth after incubation for one week, metabolites **76**, **77**, and **78** at identical concentration had only a very slight inhibitory effect. This led to the conclusion that the metabolism of camalexin (**23**) by *R. solani* was a detoxification (Pedras & Khan, 1997).

To probe the selectivity of the enzymes involved in detoxification of brassinin (**1**), the metabolism and antifungal activity of several dithiocarbamates by *P. lingam* was investigated (Pedras, Khan, Smith & Stettner, 1997a). Because methyl tryptaminedithiocarbamate (**79**) was significantly more inhibitory to *P. lingam* than brassinin (**1**), its transformation by fungal cells was examined (Pedras & Okanga, 1998c). Compounds **80** and **83** were the major metabolites obtained from fungal transformation of **79**, followed by acetyltryptamine **82** and minor components. A



Scheme 16

possible route for the metabolism of the brassinin homolog **79** by *P. lingam* is shown in Scheme 17. There appeared to be two major pathways, one leading to acid **83** via tryptamine (**81**) and acetyltryptamine (**82**) and the other leading to dithiocarbamate **80**. Interestingly, the fungal transformation of **79** occurred significantly faster in the absence of light (three to five days vs. two weeks), although a similar metabolite profile was detected. The metabolism of dithiocarbamate **79** by virulent isolates of *P. lingam* was a detoxification process which proceeded much slower than that of the naturally occurring phytoalexin **1**. That is, **1** was metabolized in 24 h to indole-3-carboxylic acid while **79** could be recovered from fungal cultures even after incubation for two weeks. The difference between the fungal metabolism of compounds **1** and **79** was attributed to either higher toxicity of **79**, or to the specificity



Scheme 17

of the enzymes involved in the transformations (Pedras & Okanga, 1998c).

7. Applications

It is of great importance to determine the antifungal activity of cruciferous phytoalexins, since from this information a 'phytoalexin blend' which could make plant defense mechanisms more effective against diverse fungal pathogens may be established. The targeted phytoalexin blend could be introduced either through genetic manipulation of biosynthetic pathways, or by screening brassica lines generated in diverse breeding programs. Furthermore, an understanding of the detoxification mechanisms employed by phytopathogens to overcome cruciferous phytoalexins should result from current biotransformation studies. Such studies may lead to the biorational design of antifungal agents selective against phytopathogens (Pedras, 1998). Results discussed above on the biotransformation of cruciferous phytoalexins indicated that different pathogens utilized different enzymes to transform the same phytoalexin and that those processes may contribute to successful plant colonization. For example, a possible strategy for deterring *P. lingam* could be the design of analogs that would inhibit the enzyme(s) involved in the detoxification of brassinin (**1**). Its analogs might have the advantage of acting synergistically with the natural disease resistance factors of plants.

Furthermore, the unusual chemical structures of cruciferous phytoalexins have suggested a new perspective of the fungal degradation of their host plants' defenses. Recent studies (Pedras & Okanga, 1998a; Pedras & Okanga, 1999) suggested the exciting possibility of establishing biosynthetic pathways of cruciferous phytoalexins in fungi, since the detoxification of cyclobrassinin (8) by different fungal species appeared to mimic biosynthetic pathways in plants. However, a better understanding will emerge upon tracing a complete map of phytoalexin transformation in both crucifers and their pathogenic fungi.

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