Strategies of fungal pathogens: detoxification of a cruciferous phytoalexin by mimicry

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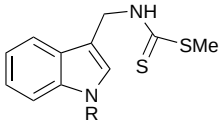
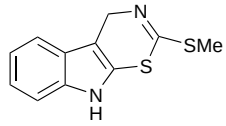
Two different isolates of the phytopathogen *Phoma lingam* metabolized and phytoalexin cyclobrassinin 3 to the phytoalexins dioxibrassinin 5 and brassilexin 9; both 5 and 9 were further metabolized and detoxified by each fungal isolate.

Phytoalexins[1] are part of the induced chemical defenses produced by plants in response to several forms of stress, including fungal attack. In spite of the enormous interest in phytoalexins, cruciferous[2] phytoalexins may have further significance due to their unique chemical structures and additional biological activity.[3] Brassinin **1**, methoxybrassinin **2**



1 R = H
2 R = OMe



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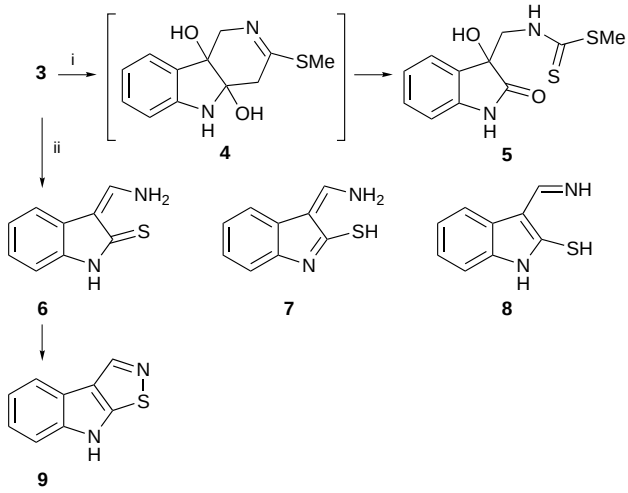
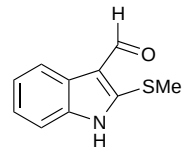
and cyclobrassinin **3** were the first reported[4] sulfur-containing phytoalexins produced by crucifers, a plant family with high sulfur requirements.[5] In general, the timing, rate of accumulation and relative amounts of phytoalexins synthesized in leaf tissues play crucial roles in plant resistance to pathogen invasion.[6] However, when pathogenic fungi can effectively disarm the plant by detoxifying phytoalexins, the outcome of the interaction can favour the pathogen and be detrimental to the plant. Although to date only a few examples demonstrate that brassica pathogens can detoxify phytoalexins efficiently,[2] multiple examples exist in other plant species.[7] We have demonstrated previously that the blackleg fungus [*Phoma lingam* (Tode ex Fr.) Desm., perfect stage *Leptosphaeria maculans* (Desm.) Ces. et de Not.], one of the most destructive pathogens of rapeseed (*Brassica napus, B. rapa*) can overcome the plant's chemical defenses by promptly transforming brassinin **1** into non-toxic indole-3-carboxylic acid.[8] Here we describe the unprecedented biotransformation of the brassica phytoalexin cyclobrassinin **3** to the phytoalexins dioxibrassinin **5** and brassilexin **9**, by the 'virulent' and 'avirulent' isolates of *P. lingam*,[9] respectively.

In initial experiments, cyclobrassinin **3**[10] was incubated with the so-called 'avirulent' isolate Unity† to establish a time-course transformation profile.[11] Analysis of organic extracts of culture samples by HPLC‡ indicated a rapid decrease in the concentration of **3** (HPLC retention time, t_r = 24.5 min) and the concurrent appearance of two additional constituents (t_r = 7.2 and 12.1 min) over a 12 h period. Subsequently, to obtain sufficient quantities of each constituent, larger-scale fungal cultures incubated with cyclobrassinin **3** were extracted with Et_2O, the extract fractionated by chromatography§ and each fraction analysed by HPLC. The fractions containing the aforementioned new constituents were analysed by standard spectroscopic methods (NMR, HRMS, FTIR, UV) for structural elucidation. Based on these results the constituent of t_r = 12.1 min was established to be the known phytoalexin brassilexin **9**,¶ whose structure we confirmed by synthesis.[2] The structure of the constituent of t_r = 7.2 min, a relatively less-stable metabolite, was assigned as a mixture of the related tautomers



Scheme 1

3-methylenaminoindole-2-thione **6**, 3-methylenaminoindole-2-thiol **7** and 3-(methylimino)indole-2-thiol **8**, based on spectroscopic data‖ and derivatization. Two days after incubation of the isolate Unity with cyclobrassinin, no brassilexin or other phytoalexins or putative metabolites were detected in any of the cultures or their extracts. The mycelial mass of cultures incubated with cyclobrassinin **3** was similar to that of control cultures. Similar experiments carried out with the 'virulent' isolate BJ-125† incubated with cyclobrassinin **3** for 12 h afforded yet another known phytoalexin, dioxibrassinin **5**[12] (t_r = 7.5 min), whose chemical structure we confirmed by synthesis. Two days after incubation of the virulent isolate BJ-125 with cyclobrassinin **3**, no phytoalexins or derivatives were detected in any of the cultures or their extracts. In addition, the mycelial mass of cultures incubated with cyclobrassinin **3** was similar to that of control cultures. These remarkable results indicate that cyclobrassinin **3** is detoxified *via* the phytoalexins brassilexin **9** or dioxibrassinin **5**, depending on the particular fungal species, as shown in Scheme 1. The reactive intermediate **4**, similar to that proposed for the biosynthesis of a brassica phytoalexin,[12] could explain the formation of **5** through enzymatic dioxygenation of **3**. Additional experiments with other fungal isolates incubated with cyclobrassinin indicated similar results within the same species. It is worthy to note that cyclobrassinin **3** has been proposed as an *in planta* biosynthetic precursor of both brassilexin **9**[13] and brassicanal A **10**.[14]



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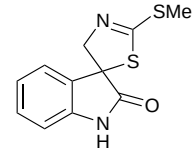

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However, *in planta*, biosynthetic relationships have only been established between brassinin **1** and cyclobrassinin **3** and brassinin **1** and spirobrassinin **11**.[12,15]

Our results suggest that two different fungal species, the so-called 'virulent' and 'avirulent' groups,† can metabolize the phytoalexin cyclobrassinin **3** 'mimicking' pathways that may operate in the plant (*i.e.* **3** to **5** and **3** to **9**, respectively).** Considering that fungal pathogens have been coevolving with plants for multiple generations, the detoxification of phytoalexins by 'mimicry' appears quite plausible. Nonetheless, because *in planta* only a part of the biosynthetic pathway of cruciferous phytoalexins has been established, such a hypothesis remains to be demonstrated. A clearer picture will eventually unfold upon tracing a complete map of phytoalexin transformation in both cruciferous plants and their pathogenic fungi.

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Footnotes and References

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† The fungal species *Phoma lingam* is subdivided in various groups (see for example ref. 9); the so-called 'avirulent' group (*e.g.* isolate Unity) is now considered a species different from that of the 'virulent' group (*e.g.* BJ-125), although no formal reclassification has been done. General procedures for fungal culturing and analyses were carried out as described in ref. 11.

‡ HPLC analysis was 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 × 200 mm) equipped with an in-line filter. Mobile phase: 75% H_2O–25% MeCN to 100% MeCN, 35 min (linear gradient), flow rate = 1.0 ml min^{-1}.

§ A clean separation of the constituents could only be achieved on C-18 reversed-phase silica gel; normal silica gel chromatography lead to decomposition of the product with t_r = 7.2 min.

¶ Brassilexin **9** was first isolated from *B. juncea* (*cf.* M. Devys, M. Barbier, I. Loiselet, T. Rouxel, A. Sarniguet, A. Kollmann and J. Bousquet, *Tetrahedron Lett.*, 1988, **29**, 6447) and later synthesized (*cf.* M. Devys and M. Barbier, *Org. Prep. Proced. Int.*, 1993, **25**, 344).

‖ The ^{1}H NMR spectrum obtained (CD_3CN or CD_2Cl_2) for the tautomeric mixture of **6** and **8** displayed a doublet of doublets (dd) at δ 8.15 attributed to (=C)–*H*(NH_2); this assignment was corroborated by proton decoupling of *H*$_2$N (δ 11.15 and 6.24). Selected *spectroscopic data* for tautomeric mixture (HPLC t_r = 7.2 min): δ_H (500 mHz, CD_2Cl_2) 11.15 (br s, side-chain NH*H*), 8.98 (br s, indolic NH), 8.15 (dd, *J* 14.8, 8.2, H-1′) 7.41 (d, *J* 7.5, H-4), 7.15–7.07 (m, H-5 to H-7, 3 H), 6.24 (br s, side-chain NH*H*); δ_C (125.8 MHz, CD_2Cl_2) 179.0, 148.9, 138.0, 128.3, 124.1, 122.2, 115.3, 109.7, 108.4; δ_C (125.77 MHz, CD_3OD) (multiple signals attributable to tautomeric mixture) 177.6, 153.7, 151.7, 148.7, 140.4, 139.5, 129.9, 126.9, 125.0, 124.9, 124.1, 123.4, 122.5, 121.6, 120.9, 119.6, 115.8, 112.9, 111.7, 110.8, 110.5, 108.6; EIMS: *m/z* 176 (M^+, 100%), 149 (46), 117 (11). CIMS: *m/z* 177 (M^+ + 1, 100%), 164 (11), 145 (13).

** It is worthy to note a few examples in which fungal pathogens produce metabolites similar or identical to those of higher plants, such as some gibberellins produced by *Gibberella fukjukuroi* and a *Phaeosphaeria* sp., and abscisic acid produced by *Cercospora rosicola*. For a recent review see, for example, P. M. Dewick, *Nat. Prod. Re.*, 1995, **12**, 507.

1 C. J. W. Brooks and D. G. Watson, *Nat. Prod. Rep.*, 1985, 427; C. J. W. Brooks and D. G. Watson, *Nat. Prod. Rep.*, 1991, 367.
2 For a recent review on cruciferous phytoalexins, see M. S. C. Pedras, A. Q. Khan and J. L. Taylor, in *Phytochemicals for Pest Control*, ed. P. A. Hedin, R. M. Hollingworth, E. P. Masler, J. Miyamoto and D. G. Thompson, *ACS Symp. Ser.*, 658, 1997, pp. 155–166 and references cited therein.
3 R. G. Meheta, J. Liu, A. Constantinou, M. Hawthorn, J. M. Pezzuto, R. C. Moon and R. Moriarty, *Anticancer Res.*, 1994, **14**, 1209. Recent studies attributed a cancer preventive role to the phytoalexins brassinin, cyclobrassinin and spirobrassinin.
4 M. Takasugi, N. Matsui and A. Shirata, *J. Chem. Soc., Chem. Commun.*, 1986, 1077.
5 R. Marquard, K. C. Walker, in *Brassica Oilseeds*, ed. D. Kimber and D. I. McGregor, CAB International, Wallingford, UK, 1995, pp. 195–213.
6 C. J. Smith, *New Phytol.*, 1996, **132**, 1; W. Barz, W. Bless, G. Börger-Papendorf, W. Gunia, V. Mackenblock, D. Meier, C. Otto and E. Süpei, in *Bioactive Compounds from Plants*, Ciba Foundation Symposium, Wiley, Chichester, England, 1990, pp. 140–156.
7 *Handbook of Phytoalexin Metabolism and Action*, ed. M. Daniel and R. P. Purkayastha, Marcel Dekker Inc., New York, 1995, p. 650; H. D. VanEtten, D. E. Mathews and P. S. Mathews, *Annu. Rev. Phytopathol.*, 1989, **27**, 143.
8 M. S. C. Pedras and J. L. Taylor, *J. Org. Chem.*, 1991, **56**, 2619; M. S. C. Pedras, I. Borgmann and J. L. Taylor, *Phytochem.* (*Life Sci. Adv.*), 1992, **11**, 1; M. S. C. Pedras and J. L. Taylor, *J. Nat. Prod.*, 1993, **56**, 731.
9 M. S. C. Pedras, J. L. Taylor and V. M. Morales, *Phytochemistry*, 1995, **38**, 1215; J. L. Taylor, M. S. C. Pedras and V. M. Morales, *Trends Microbiol.*, 1995, **3**, 202.
10 M. Takasugi, K. Monde, N. Katsui and A. Shirata, *Bull. Chem. Soc. Jpn.*, 1988, **61**, 285.
11 M. S. C. Pedras and A. Q. Khan, *J. Agric. Food Chem.*, 1996, **44**, 3403.
12 First isolation: K. Monde, K. Sasaki, A. Shirata and M. Takasugi, *Phytochemistry*, 1991, **30**, 2915. For synthesis, see K. Monde, M. Takasugi and T. Ohnishi, *J. Am. Chem. Soc.*, 1994, **116**, 6650.
13 M. Devys and M. Barbier, *Z. Naturforsch., Teil C*, 1992, **47**, 318.
14 K. Monde, A. Tanaka and M. Takasugi, *J. Org. Chem.*, 1996, **61**, 9053.
15 K. Monde and M. Takasugi, *J. Chem. Soc., Chem. Commun.*, 1991, 1582.

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