Probing the Phytopathogenic Blackleg Fungus with a Phytoalexin Homolog

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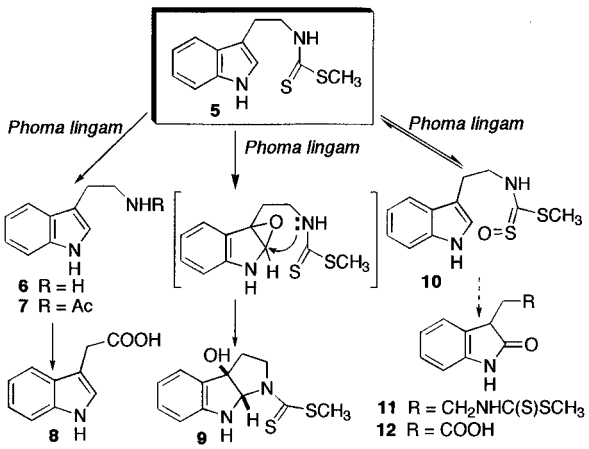
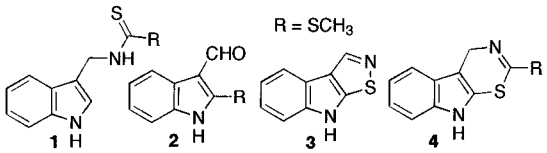
Plants produce a vast array of chemical defenses, some of which are synthesized *de novo* in response to biotic and abiotic stress, i.e., phytoalexins.[1] The detoxification of phytoalexins is a mechanism of virulence in diverse phytopathogenic fungi.[2] This mechanism allows fungi to invade plants, which may lead to significant tissue damage and concomitant yield losses. An environmentally attractive strategy to control such plant pathogens could involve application of selective inhibitors of those fungal enzymes utilized in the detoxification of phytoalexins. In this connection, we have established that the phytoalexin brassinin (**1**) is metabolized[3] and detoxified[4] by the virulent blackleg fungus [*Phoma lingam* (Tode ex Fr.) Desm., asexual stage of *Leptosphaeria maculans* (Desm.) Ces. et de Not.], one of the most important pathogens of the oilseeds rapeseed (*Brassica napus, B. rapa*), canola (*B. napus, B. rapa*), and mustard (*B. juncea*).[5] In addition, the dithiocarbamate group of brassinin (**1**) was thought to be essential for its antifungal activity. Nevertheless, despite the relatively higher antifungal activity of mono- and dichlorobenzyl dithiocarbamates, their detoxification by virulent isolates of the blackleg fungus was not averted.[6] We have also demonstrated that this pest can detoxify other brassica phytoalexins, including brassicanal A (**2**),[7] brassilexin (**3**),[8] and cyclobrassinin (**4**)[9] via different pathways, suggesting that its efficacy in disarming host plants has a broad enzymatic base.[10] To further probe the selectivity of the enzymes involved in detoxification of brassinin (**1**), the metabolism and antifungal activity of phytoalexin analogues are being investigated. Because methyl tryptaminedithiocarbamate (**5**) was significantly more inhibitory to *P. lingam* than brassinin, we examined its transformation by fungal cells. Here we disclose unique chemical aspects of the metabolic pathway leading to the fungal detoxification of **5**, as well as the unprecedented in vitro rearrangement of the dithiocarbamate *S*-oxide **10** to the 2-oxindole derivative **11**.

Scheme 1





Preliminary experiments were carried out to determine the time required for complete metabolism of dithiocarbamate **5** by virulent isolates of *P. lingam*. Cultures were incubated with **5**,[11] samples were withdrawn at 0−24 h intervals, the mycelia were filtered off, and the broth was extracted with Et_2O. Extracts were analyzed by TLC and HPLC to determine the optimum incubation time for isolation of possible metabolic intermediates. Subsequently, fractionation of Et_2O extracts obtained from larger scale cultures afforded compounds **6**−**12** (Scheme 1).[12] Compounds **8** and **9** were the major metabolites obtained from fungal transformation of **5**, representing ca. 65% of the total amount of isolated metabolites, followed by acetyltryptamine **7** (15%) and minor components **6** and **10**−**12** (less than 5%). The structures of compounds **6**−**8** were readily determined by comparison of their spectroscopic data with those of authentic samples.[13] The structures of all other metabolites were deduced from analyses of their spectroscopic data as discussed below and corroborated by synthesis.

The HRMS analyses of **9**−**11** indicated for each compound a molecular formula of $C_{12}H_{14}N_2OS_2$, which formally represented addition of one oxygen unit to dithiocarbamate **5**. The ^{1}H NMR spectra and optical rotations of both **9** and **11** suggested that they were structural isomers (four magnetically nonequivalent methylene protons) and chiral. The ^{1}H NMR spectrum of **10** displayed resonances comparable to those of **5** (within 0.1 ppm), except for the SCH_3 (δ 2.35 for **10** vs δ 2.56 for **5**) and CH_2N (δ 3.80 for **10** vs δ 4.06 for **5**) groups. Furthermore, the ^{13}C NMR spectrum of **10** was similar to that of **5** (within 2 ppm) except for resonances

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(9) First isolation of cyclobrassinin (**4**) see ref 3. Metabolism of cyclobrassinin: Pedras, M. S. C.; Okanga, F. I. *Chem. Commun.*, in press.

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(11) Dithiocarbamate **5** (higher antifungal activity than brassinin (**1**)) was administered to cultures of virulent isolates of *P. lingam* at lower concentration (final concentrations 1.6×10^{-4} M and 2.0×10^{-4} M) than brassinin (final concentration 5.0×10^{-4} M).

(12) Cultures were incubated with **5** up to 3 weeks; **6**, **7**, and **9**−**11** were detected in cultures after 24 h of incubation, whereas **8** and **12** were detected after 5 days of incubation.

(13) Authentic samples of **6** and **8** were purchased from Aldrich Chemical Co.; **7** was obtained upon standard acetylation of **6** (Ac_2O/Py).

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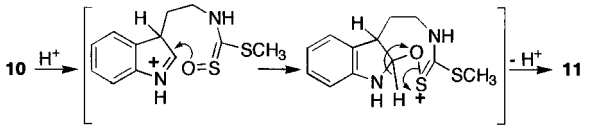
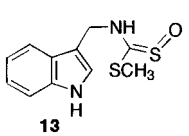
Scheme 2



attributable to thiocarbonyl (δ_C 194.5 in **10** vs δ_C 198.8 in **5**) and SCH_3 (δ_C 13.3 in **10** vs δ_C 18.1 in **5**) groups. These results and the FTIR spectrum of **10** (905 cm^{-1}) indicated the presence of an *S*-oxide group.[14] On the other hand, comparison of the NMR spectral data of **9** and **11** with those of **5** indicated that the structural differences resided in the pyrrole moieties.[15] Compared with **5**, the ^1H NMR spectrum of **9** displayed signals for only 12 protons and the ^{13}C NMR spectrum indicated two sp^3 carbons (δ_C 87.2, and 86.2) instead of two sp^2 carbons in **5**. These results, together with 2D-NMR spectral data, suggested a pyrroloindole ring system for compound **9**. Similarly, the 2-oxindole substructure of dithiocarbamate **11** was deduced from NMR spectral data. The structure of the minor acid **12** followed also from spectroscopic data. Compounds **9**−**11** do not appear to have been previously described; the absolute configurations of **9** and **11** remain to be determined. The chemical structures of metabolites **9**−**12** were confirmed unambiguously by chemical synthesis, of which the conversion of *S*-oxide **10** to oxindole **11** is particularly noteworthy.

We have previously demonstrated that the unusual brassinin *S*-oxide (**13**), although rather unstable at room temperature, can be prepared in modest yield (ca. 30%) by reaction of dithiocarbamate **1** with *m*-CPBA;[16] similar reaction of **5** with *m*-CPBA yielded *S*-oxide **10** in ca. 50% yield. Surprisingly, **10** rearranged to oxindole **11** on standing in $CDCl_3$ solution at room temperature.[17] This transformation proceeded much slower in CH_2Cl_2 or $CHCl_3$ but at a similar rate in CH_2Cl_2 in the presence of TsOH. Furthermore, oxidation of **5** with *m*-CPBA, followed by acidification of the reaction mixture with TsOH, yielded oxindole **11** directly. These results suggest that formation of **11** might be initiated by protonation of the indole ring at C-3,[18] followed by intramolecular oxygen transfer from C═S═O to C-2 with regeneration of the dithiocarbamate group (Scheme 2). Compounds containing a dithiocarbamate *S*-oxide group are rarely isolable and have been characterized mostly as mixtures.[19] Structures similar to **10** (i.e., *S*-oxide **13**) appear to have been described only once.[3,4] However, the rearrangement observed for *S*-oxide **10** was not noted with **13**, which decomposed to brassinin (**1**) and undetermined products on standing. Although the decomposition of certain dithiocarbamate *S*-oxides appears to lead mainly to the thiol carbamates (sulfur extrusion), particularly in the presence of acid,[19] we have not isolated such products from **10** or **13**. The relatively higher stability of dithiocarbamate **5** and the corresponding *S*-oxide **10** in relation to brassinin (**1**) and *S*-oxide **13** is attributed to the additional CH_2 group, which reduces the propensity for elimination of dithiocarbamic acid or equivalent (cf. **14**). The photosensitized cyclization of dithiocarbamate **5** to pyrroloindole **9** was achieved in 70% yield, following a procedure previously reported[20] for the corresponding methyl carbamate. A similar oxygenation of N_b-acetyltryptamine yielded the corresponding pyrroloindole product in lower yield.[21] Although these photooxygenation reactions emphasized the likelihood of having similar processes occurring nonenzymatically, incubation of **5** or **7** under conditions identical with those used for fungal cultures but in the absence of the fungus, followed by HPLC analysis of sample extracts indicated that both **5** and **7** were stable for at least two weeks. Finally, in vitro oxidation of acid **8** with DMSO/HCl yielded acid **12**.[18a] Although a similar reaction could explain the presence of **12** in fungal cultures incubated with **5**, control experiments indicated that such a process did not occur under the experimental conditions utilized for fungal metabolism of **5**.



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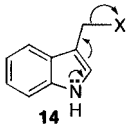

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A route for the metabolism of the brassinin homologue **5** by *P. lingam* is proposed in Scheme 1. There appear to be two major pathways, one leads to acid **8** via tryptamine (**6**) and acetyltryptamine (**7**) and the other yields dithiocarbamate **9**; neither **8** or **9** appeared to be metabolized further. In addition, a minor pathway may lead to products **11** and **12** (≤1%). Although **11** appeared as a likely precursor of acid **12**, we have been unable to observe the fungal transformation of **11** into **12**. Surprisingly, although **5** is significantly more inhibitory to *P. lingam* than **10**, *S*-oxide **10** administered to fungal cultures was reduced to mostly **5**, in less than 48 h. Control experiments indicated that **10** was stable under those incubation conditions for several days, suggesting that the transformation of **10** to **5** was an enzymatic process. In additional experiments, we determined that the fungal transformation of **5** occurred significantly faster in the absence of light (3−5 days vs 2 weeks), with a similar metabolite profile being detected. Finally, we determined that none of the metabolic products **8**−**12** showed noticeable inhibitory activity against *P. lingam*.

In conclusion, the metabolism of dithiocarbamate **5** by virulent isolates of *P. lingam* is a detoxification process; however, this process proceeded much slower than that of the naturally occurring homologue **1**. Both fungal degradation pathways of dithiocarbamates **1** and **5** yielded carboxylic acids, none of which appeared to be inhibitory against *P. lingam*. Clearly, further work must be carried out to probe the specificity of the detoxifying enzymes and to understand the intriguing effect of light on the rate of the fungal transformation of **5**. Nonetheless, our results strongly suggest that phytoalexin analogues can be designed to deter fungal phytopathogens more effectively than phytoalexins. Moreover, such analogues might have the advantage of acting synergistically with the natural disease resistance factors of plants.

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Supporting Information Available: Synthesis and spectroscopic data for **5**, **9**, **10**, and **11** (3 pages).

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(14) Compound **10** could exhibit *E*/*Z* isomerism, as reported previously for brassinin *S*-oxide.[3] Nevertheless, considering the sharpness of its NMR resonances, it appeared that only one isomer was present and an *E* stereochemistry would allow intramolecular hydrogen bonding.

(15) Spectroscopic data are in complete agreement with the proposed structures. See the Supporting Information.

(16) Prepared as reported in ref 4.

(17) Compound **11** was isolated in 40% yield together with **5** (20%) and polar unidentified products.

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