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UNPRECEDENTED DETOXIFICATION OF THE CRUCIFEROUS PHYTOALEXIN CAMALEXIN BY A ROOT PHYTOPATHOGEN

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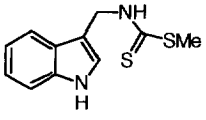
Abstract: The unusual metabolism of the phytoalexin camalexin by the root rot fungus *Rhizoctonia solani* Kuhn is reported. This transformation proceeded via 5-hydroxycamalexin, further transformed into 5-hydroxyindole-3-carbonitrile and 5-hydroxy-2-formamidophenyl-2'-thiazolylketone. Most importantly, the metabolites resulting from transformation of camalexin were significantly less toxic to the fungus than camalexin. © 1997 Elsevier Science Ltd.

Phytoalexins[1] play a significant role in the response of plants to pathogen attack. These secondary metabolites, which are synthesized de novo by plants in response to diverse forms of stress, including fungal infection, are part of the plant's chemical and biochemical defense mechanisms.[2] However, when pathogenic fungi can effectively disarm the plant by detoxifying phytoalexins, the outcome of the interaction can favor the pathogen and be detrimental to the plant.

Phytoalexins from crucifers (broccoli, cabbage, canola, cauliflower, rapeseed, turnip, etc.) are structurally (e.g., **1–5**) and biogenetically related, but display significantly different biological activities.[3] For example, brassinin (**1**), one of the first reported cruciferous phytoalexins produced by a variety of *Brassica* species,[4] and camalexin (**5**), also a cruciferous phytoalexin produced by *Arabidopsis thaliana*[5] and *Camelina sativa*,[6] have significantly different antifungal activity. While brassinin is active against the blackleg fungus[7] (*Leptosphaeria maculans*, asexual stage *Phoma lingam*) of rapeseed/canola (*Brassica napus*, *B. rapa*)[8-11] camalexin appears to have no detectable inhibitory effect.[3] Nevertheless, there is wide evidence suggesting that camalexin has an important role in plant defense. Phytoalexin-deficient mutants of *A. thaliana* that accumulated low levels of camalexin displayed increased susceptibility to *Perenospora parasitica* (fungal pathogen).[12] These Arabidopsis mutants also indicated that camalexin inhibited *in planta* the growth of virulent *Pseudomonas syringae* (a bacterial phytopathogen) strains, but not the growth of avirulent *P. syringae*.[13] In addition, the resistance of false flax (*C. sativa*) to *R. solani*[14] and blackspot (*Alternaria brassicae*, (Berk.) Sacc.)[15] fungi was directly correlated with the production of camalexin. On the other hand, camalexin caused cell death on *A. thaliana* and *P. syringae* at concentrations higher than 250 μg/mL.[16]



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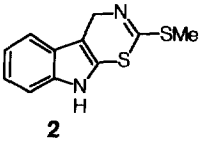

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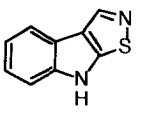

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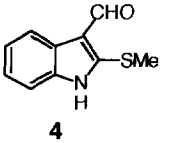

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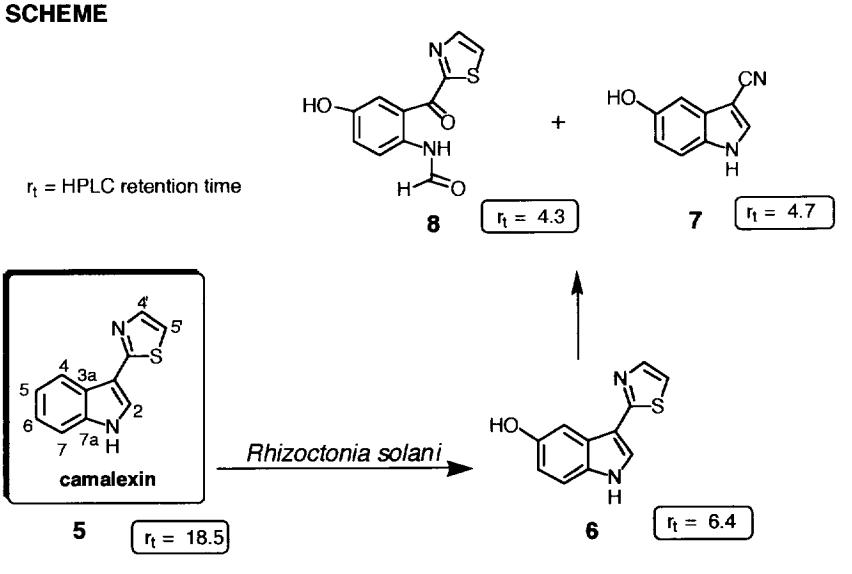
Although to date only a few examples demonstrate that fungal pathogens can detoxify cruciferous phytoalexins efficiently[3,17] multiple examples exist in other plant species[18] suggesting that phytopathogens

can effectively overcome the plants' chemical defenses. We have established that the blackleg fungus can metabolize and detoxify brassinin (**1**),[9–11] cyclobrassinin (**2**),[19] brassilexin (**3**),[3] and brassicanal A (**4**);[8] however, camalexin (**5**)[3] was not metabolized by either virulent or avirulent isolates[20] of the blackleg fungus. Reasoning that camalexin might be metabolized by virulent fungi capable of infecting false flax, (i.e., a plant that produces camalexin) and considering that the root rot fungus *Rhizoctonia solani* Kuhn can infect false flax, we examined camalexin metabolism by this flax pathogen. Here we wish to report the unprecedented metabolism of camalexin by the plant pathogen *R. solani,* as well as the biological activity of the intermediates of this pathway.

Preliminary experiments established the minimum inhibitory concentration (50 mg/L, 2.5×10^{-4} M) of camalexin (**5**) to *R. solani*. Subsequently, camalexin was incubated with *R. solani* at concentrations that would allow mycelial growth (25 mg/L, 1.2×10^{-4} M). Culture samples were withdrawn at 0–24 h intervals, and extracted first with Et_2O, then acidified and reextracted. Separate cultures containing only the fungus or only the medium containing camalexin were incubated under similar conditions and extracted. Comparison of HPLC chromatograms[21] of neutral extracts of fungal cultures incubated with camalexin and those of control cultures indicated that camalexin was completely metabolized in 72 h. In addition, the HPLC analyses indicated the optimum incubation time for isolation of the putative intermediates/products of camalexin metabolism.[22] Isolation of the components (prep TLC) of neutral Et_2O extracts obtained from larger scale cultures of *R. solani* incubated with camalexin (**5**) yielded metabolites **6**-**8**. The chemical structure of each metabolite was deduced from comparison of their spectroscopic data with those of camalexin, and confirmed by chemical syntheses.

SCHEME



Relative to camalexin ($C_{11}H_8N_2S$), metabolite **6** contained an additional oxygen atom ($C_{11}H_8N_2OS$), as determined by HR-EIMS. The ^1H NMR spectrum of **6** displayed a three-proton spin system (7.49, d, J = 2.0 Hz; 7.30, d, J = 8.7 Hz; 6.77, dd, J = 8.7; 2.0 Hz) in addition to H-2 and two thiazole protons, suggesting the presence of an indole substituent located either at C-5 or C-6.[23] The ^{13}C NMR spectrum of **6** displayed 11 carbon signals, one of which indicated a deshielded sp^2 C atom (153.3 ppm). These spectroscopic features indicated that metabolite **6** contained a OH group attached to C-5 or C-6. That the OH group was attached to C-5 rather than C-6 was finally deduced from comparison of HMBC and HMQC spectral data with those of camalexin. Furthermore, the structural assignment was corroborated by synthesis of 5-hydroxycamalexin (**6**) following a route similar to that of camalexin.[24]

Unlike 5-hydroxycamalexin (**6**), metabolites **7** and **8** did not appear to accumulate to any significant extent in cultures of *R. solani* incubated with camalexin (**5**); the yields of both metabolites **7** and **8** were rather low and posed a significant problem to the elucidation of their structures; from a 2-liter culture of *R. solani* containing ca. 50 mg of camalexin, only 2 mg of each metabolite could be isolated. Nevertheless, the structure of nitrile **7** was readily deduced from analysis of its spectroscopic data,[25] and confirmed by synthesis.[26] The ^1H NMR spectrum of **8** indicated spin systems similar to those of 5-hydroxycamalexin (**6**); the molecular formula $C_{11}H_8N_2O_3S$ obtained by HR-EIMS indicated the presence of two additional oxygen atoms relative to **6**. Unlike **6**, the ^{13}C NMR spectrum of **8** displayed two resonances indicative of C=O groups (or equivalents, at 187.1 and 161.9 ppm). Furthermore, while HMQC spectral data suggested that the thiazole ring of metabolite **8** was intact, a carbon signal at 161.9 ppm attached to a singlet proton at 8.19 ppm indicated that the indole ring had been oxidized.[27] Incidentally, a compound related to **8** (having at C-5 a H rather than OH) had been reported in a previous synthesis of camalexin,[24] and thus **8** was synthesized utilizing a similar route. Finally, comparison of the spectroscopic data of our synthetic material and those of the compound obtained from fungal cultures confirmed the structural assignment of **8** unambiguously.

To confirm the sequence of the biotransformation steps, compounds **6**, **7**, and **8** (final concentration 1.2×10^{-4} M) were separately administered to cultures of *R. solani*. The cultures were incubated and samples withdrawn at 0-24 h intervals, extracted, and analyzed by TLC and HPLC. As expected, **7** and **8** were detected in the cultures incubated with **6** after 24 h, with complete transformation of **6** to **7** and **8** occurring within 9 days. No further products of this metabolism were detected. The proposed sequence of reactions that camalexin (**5**) undergoes when incubated with virulent *R. solani* is shown in the scheme. The first biotransformation step of camalexin (**5**) is a simple oxidation of its indole ring to yield **6**; this compound was detected 24 h after incubation of camalexin with *R. solani*. Unexpectedly, however, the next transformation step led to oxidative bond cleavage of the indole ring, yielding compound **8**. This is the first time that oxidation of the indole ring of a cruciferous phytoalexin has been observed; our previous work with other phytoalexins indicated that fungal oxidation occurs at the indole substituents at C-2 or C-3. The biodegradation of 5-hydroxycamalexin (**6**) to nitrile **7** could involve elimination of sulfur and acetylene. 5-Hydroxycamalexin (**6**) is a new compound, although the methylated derivative 5-methoxycamalexin, and the 6-methoxy analog as well, were previously synthesized.[28] It is worthy to note that the antifungal activity of the 5-methoxy derivative was higher than that of the 6-methoxy derivative on a *Cladosporium* sp.; however, in agreement with our observations, camalexin was

significantly more active than either of these two derivatives.[28] Likewise, **7** and **8** have not been previously described, although indole-3-carbonitrile has been previously isolated from a halophilic bacterium.[29]

The low recovery of metabolites **7** and **8** from cultures of *R. solani* incubated with camalexin could be due to biodegradation of these compounds by the fungus. We investigated this possibility by adding each compound separately to fungal cultures; however, **8** was quantitatively recovered even after one to two weeks of incubation, and **7** was metabolized to undetermined products over a three week period. Thus, further metabolism of **7** could account for its low recovery. In addition, HPLC analysis of extracts of fungal cells indicated that only traces (<1%) of **6**, **7**, and **8** were present in the cells at any given time. In conclusion, **6** is the major metabolite (ca. 90% after three days of incubation) of camalexin, clearly resulting from simple oxidation of **5**, and **7** and **8** result from slow biotransformation of **6**.

Of much greater consequence is the fungal oxidation of camalexin (**5**) to yield significantly less toxic products. While agar plates[30] containing camalexin at 5×10^{-4} M inoculated with *R. solani* showed no mycelial growth after incubation for one week, metabolites **6**, **7**, and **8** at identical concentration had only a very slight inhibitory effect (ca. 2% relative to control cultures). In conclusion, the metabolism of camalexin by *R. solani* is a detoxification process that allows the pathogen to overcome a plant's chemical defense. The demonstration of this detoxification process is of great importance, not only because it allows a better understanding of an interaction between plant and pathogen, but also because this process can be exploited to deter pathogens. That is, the detoxification of phytoalexins by fungal pathogens could be utilized to control pathogenic fungi, if inhibitors of those fungal detoxifying enzymes were available.[3] The results we obtained with camalexin, in conjunction with our earlier work,[3,8–11] indicate that phytoalexins are effective against specific pathogens, having no detectable effects on other pathogens that may also attack the same plant. Thus, it is likely that antifungal compounds related to camalexin but with additional structural features might selectively inhibit *R. solani* and at the same time have no deleterious effects on other organisms. This would be an environmentally advantageous strategy for controlling this root pathogen and possibly other pathogenic fungi of crucifers.

Acknowledgments

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References and Notes

1. (a) Brooks, C. J.; Watson, D. G. *Nat. Prod. Rep.* **1985**, 427; (b) Brooks, C. J.; Watson, D. G. *Nat. Prod. Rep.* **1991**, 367.
2. (a) Dixon, R. A. *Biol. Rev.* **1986**, *61*, 239; (b) Dixon, R. A.; Harrison, M. J.; Lamb, C. J. *Annual Rev. Plant Phytopathol.* **1994**, *32*, 479; (c) Kuc, J. *Annual Rev. Phytopathol.* **1995**, *33*, 275; (d) Smith, C. J. *New Phytol.* **1996**, *132*, 1.

3. For a recent review on brassica phytoalexins: Pedras, M. S. C.; Khan, A. Q.; Taylor, J. L. In *Phytochemicals for Pest Control*; Hedin, P. A.; Hollingworth, R. M.; Masler, E. P.; Miyamoto, J.; Thompson, D. G. Eds.; ACS Symposium Series 658, 1997; pp 155-166.
4. (a) Takasugi, M.; Katsui, N.; Shirata, A. *J. Chem. Soc., Chem. Commun.* **1986**, 1077; (b) Takasugi, M.; Monde, K.; Katsui, N.; Shirata, A. *Bull. Chem. Soc. Jpn.* **1988**, *61*, 285.
5. Tsuji, J.; Jackson, E. P.; Gage, D. A.; Hammerschmidt, R.; Somerville, S. C. *Plant Physiol.* **1992**, *98*, 1304.
6. Browne, L. M.; Conn, K. L.; Ayer, W. A.; Tewari, J. P. *Tetrahedron* **1991**, *47* (24), 3909.
7. For a review on the blackleg the fungus see, for example, Pedras, M. S. C.; Séguin-Swartz, G. *Can. J. Plant Pathol.* **1992**, *14*, 67.
8. Pedras, M. S. C.; Khan, A. Q. *J. Agric. Food Chem.* **1996**, *44*, 3403.
9. Pedras, M. S. C.; Borgmann, I; Taylor, J. L. *Phytochem. (Life Sci. Adv.)* **1992**, *11*, 1.
10. Pedras, M. S. C.; Taylor, J. L. *J. Org. Chem.* **1991**, *56*, 2619.
11. Pedras, M. S. C.; Taylor, J. L. *J. Nat. Prod.* **1993**, *56*, 731.
12. Glazebrook, J.; Zook, M.; Mert, F.; Kagan, I.; Rogers, E. E.; Crute, I. R.; Holub, E. B.; Hammerschmidt, R.; Ausubel, F. M. *Genetics* **1997**, *146*, 381.
13. (a) Ausubel, F. M.; Katagiri, F.; Mindrinos, M.; Glazebrook, J. *Proc. Natl. Acad. Sci. U.S.A.* **1995** *92*, 4189; (b) Glazebrook, J.; Rogers, E. E.; Ausubel, F. M. *Genetics* **1996**, *143*, 973.
14. Conn, K. L.; Browne, L. M.; Tewari, J. P.; Ayer, W. A. *J. Plant Biochemistry & Biotechnoly* **1994**, *3*, 125.
15. Jejelowo, O. A.; Conn, K. L.; Tewari, J. P. *Mycol. Res.* **1991**, *95*, 928.
16. Rogers, E. E.; Glazebrook, J.; Ausubel, F. M. *Molecular Plant-Microbe Interaction* **1996**, *9*, 748.
17. Rouxel, T.; Kolman, A.; Ballesdent, M. H. In *Handbook of Phytoalexin Metabolism and Action*; Daniel, M.; Purkayastha, R. P., Eds.; Marcel Dekker, Inc., New York, N.Y. **1995**, pp 229-261.
18. (a) *Handbook of Phytoalexin Metabolism and Action*; Daniel, M.; Purkayastha, R. P. Eds. Marcel Dekker, Inc.; New York, N.Y. 1995; pp 650; (b) Van Etten, H. D.; Mathews, D. E.; Mathews, P. S. *Annu. Rev. Phytopathol.* **1989**, *27*, 143.
19. Pedras, M. S. C.; Okanka, F. I., submitted.
20. Virulent and avirulent blackleg fungi are now considered two different species; see *inter alia*, Pedras, M. S. C.; Taylor, J. L.; Morales, V. M. *Phytochemistry* **1995**, *38*, 1215.
21. HPLC analysis: was carried out with a high performance Hewlett–Packard liquid chromatograph equipped with quaternary pump, automatic injector, and diode array detector (wavelength range 190–600 nm), degasser, and 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 of 1.0 mL/min.
22. Typical metabolism experiment: *Rhizoctonia solani*, virulent isolate AG 2-1 and weakly virulent isolate AG 4 were utilized; the fungal isolates were grown on potato dextrose agar (PDA) plates at 24 ± 2 °C, under continuous light for 5 to 6 days. Liquid cultures were initiated by inoculating PD liquid medium with five agar plugs cut from edges of 5-day-old solid cultures. Solutions of camalexin (**5**), or the compound under investigation (in DMSO) were administered to 48–h–old liquid cultures (final concentration 1.2×10^{-4}) and to uninoculated media. Cultures were incubated on a shaker at 130 rpm, at 24 ± 2 °C. Samples (5–10 mL) were withdrawn every 6 h for 24 h, and every 24 h up to two weeks, and were either immediately frozen or filtered and extracted with Et_2O,[8] followed by butanol. All the compounds were stable in uninoculated potato dextrose medium for at least 15 days. Compounds **6**–**8** were isolated from neutral Et_2O extracts. No other metabolites derived from camalexin were detected in

acidic or butanol extracts. The structures of the products were determined from analyses of the spectroscopic data (NMR, MS, FTIR, UV) of the purified (prep TLC) metabolites, and confirmed by chemical synthesis.

23. 5-Hydroxycamalexin (**6**): ^{1}H NMR (500 MHz, CD_3OD) δ 7.90 (H-2, br s), 7.73 (H-4', d, J = 3.4 Hz), 7.49 (H-4, d, J = 2.0 Hz), 7.42 (H-5', d, J = 3.4 Hz), 7.30 (H-7, d, J = 8.7 Hz), 6.77 (H-6, dd, J = 8.7, 2.0 Hz); ^{13}C NMR (125.5 MHz, CD_3OD) δ 166.2, 153.3, 142.6, 133.1, 127.1, 126.6, 116.7, 113.7, 113.6, 111.4, 105.1; FTIR ν_{max} 3307, 2923, 1581, 1543, 1472, 1444, 1243, 1205 cm^{-1}; EIMS, mass (relative intensity) = measured 216.0364 (M^+, $C_{11}H_8N_2OS$, calcd 216.0357) (100), 158 (33), 149 (25); CIMS, mass (relative intensity) = 217 (M^++1) (100).
24. Furstner, A.; Ernst, A. *Tetrahedron* **1995**, *51*, 773.
25. 5-Hydroxyindole-3-carbonitrile (**7**): ^{1}H NMR (500 MHz, CD_3OD) δ 7.81 (H-2, s), 7.30 (H-7, d, J = 8.7 Hz), 6.97 (H-4, d, J = 1.4 Hz), 6.82 (H-6, dd, J = 1.4, 8.7); ^{13}C NMR (125.5 MHz, CD_3OD) δ 154.3, 134.5, 131.6, 129.8, 117.8, 115.1, 114.3, 103.7, 85.4; FTIR ν_{max} 3325, 3225, 2220, 1204 cm^{-1}; EIMS, mass (relative intensity) = measured 158.0478 (M^+, $C_9H_6N_2O$, calcd 158.0480) (100), 129 (11), 103 (11); CIMS, mass (relative intensity) = 176 (M^+ + 18) (100).
26. Compound **7** was obtained from 5-hydroxyindole, after formylation[3] and conversion of the resulting aldehyde to the nitrile using hydroxylamine hydrochloride and selenium dioxide, according to Sosnovsky, G.; Krogh, J. A.; Umhoefer, S. G. *Synthesis* **1979**, 722.
27. 5-Hydroxy-2-formamidophenyl-2'-thiazolylketone (**8**): ^{1}H NMR (500 MHz, CD_3OD): δ 8.19 (H-2, s), 8.10 (H-5', d, J = 3.0 Hz), 8.04 (H-4', d, J = 3.0 Hz), 7.75 (H-4, d, J = 2.8 Hz), 7.70 (H-7, d, J = 8.8 Hz), 7.02 (H-6, dd, J = 8.8, 2.8 Hz); ^{13}C NMR (125.5 MHz, CD_3OD) δ 187.1, 168.8, 161.9, 155.2, 145.9, 130.2 (2 C's), 125.8, 124.1, 121.4, 119.8; FTIR ν_{max} 3314, 3090, 1672, 1522, 1383, 1304, 1234, 827 cm^{-1}; EIMS, mass (relative intensity) = measured 248.0254 (M^+, $C_{11}H_8N_2O_3S$, calcd 248.0256) (28), 220 (18), 192 (100), 148 (18), 149 (17), 108 (12); CIMS, mass (relative intensity) = 249 (M^+ + 1) (100).
28. Ayer, W. A.; Craw, P. A.; Ma, Y.; Miao, S. *Tetrahedron* **1992**, *48*, 2919.
29. Fu, X; Schmitz, F. J.; Tanner, R. S. *J. Nat. Prod.* **1995**, *58*, 1950.
30. Typical fungal radial growth assay: a DMSO solution (final concentration ≤ 1%) of the compound to be tested (final concentration of each compound 5×10^{-4}, 2.5×10^{-4}, 1.2×10^{-4}, and 6.2×10^{-5} M) was added to PDA medium at ca. 50 °C, mixed quickly and poured onto Petri plates. An agar plug (5 mm diameter) cut from edges of 5-day-old solid cultures of *R. solani* was placed upside down on the center of each plate, the plates sealed with parafilm, and incubated under constant light at 24 ± 2 °C for four days. The diameter of the mycelial covered area was measured daily. Control plates containing only PDA medium or PDA medium and DMSO were prepared and incubated similarly. Each assay was repeated at least twice, in triplicate.

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