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THE CAMALEXINS: NEW PHYTOALEXINS PRODUCED IN THE LEAVES OF *CAMELINA SATIVA* (CRUCIFERAE).

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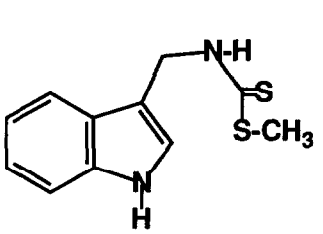
Abstract:

Two new thiazoyl substituted indole phytoalexins, camalexin (**5**) and methoxycamalexin (**8**), were isolated from *Camelina sativa* leaves following elicitation by the fungus *Alternaria brassicae*.

The phytoalexin theory of disease resistance in plants, first proposed by Müller and Börger[1], explains many plant-pathogen interactions. Recently, we and others have demonstrated that plants of the family Cruciferae accumulate phytoalexins after exposure to microorganisms. These compounds are of special interest since they were the first phytoalexins reported to contain sulfur. When *Pseudomonas cichorii* (Swingle) Stapp was used as an elicitor, the Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) produced brassinin (**1**) and cyclobrassinin (**2**)[2], and the Japanese vegetable, daikon (*Rhaphanus sativus* L. var. *hortensis*), produced spirobrassinin (**3**)[3]. On the other hand, the mustard plant (*B. juncea* (L.) Cosson) showed resistance to the blackleg fungus *Leptosphaeria maculans* (Desm.)Ces. *et* de Not. and this phenomenon was shown to be due to the synthesis and accumulation of brassilexin (**4**)[4] after infection. Recently Conn *et al*[5] have shown that leaves of false flax (*Camelina sativa* (L.) Crantz) were resistant to *Alternaria brassicae* (Berk.) Sacc., the causal agent of blackspot disease of rapeseed, and this resistance was attributed to the production of unidentified phytoalexins This paper describes the isolation, separation, and structure elucidation of the major component produced by *C. sativa* which is fungitoxic to *A. brassicae*[5].



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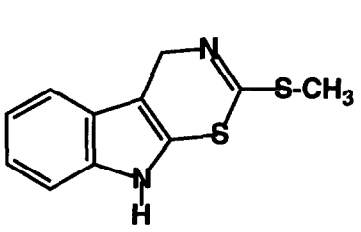

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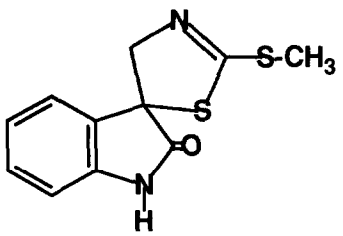

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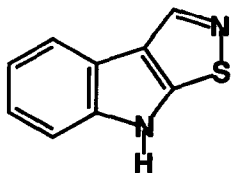

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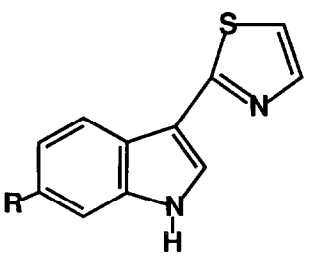
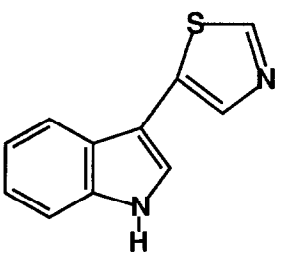
Phytoalexins produced by the leaves of *C. sativa* in response to *A. brassicae* were extracted with aqueous methanol. After concentration of the leaf extract, two antifungal metabolites were detected by bioautography[6] using *Cladosporium* sp. as the test organism. Thin layer chromatographic analysis of the concentrated extract revealed two bioactive components, one of which is strongly fluorescent under ultraviolet light and gives a positive test when sprayed with Dragendorff's reagent. Accordingly, the concentrated extract was partitioned to give a basic fraction which was separated further by vacuum liquid chromatography (VLC) over silica gel[7,8] A very small quantity of an active component, $R_f = 0.43$ ($CHCl_3$-CH_3OH 49:1), was isolated along with the major fluorescent bioactive component, $R_f = 0.33$ ($CHCl_3$-CH_3OH 49:1). Careful VLC of the major component using silica gel revealed that early and late fractions collected in the chromatography showed different charring characteristics and had different UV maxima. However, analysis of the crude component by reverse phase high pressure liquid chromatography showed a single peak when early and late fractions were analyzed. It was established that the component was indeed a mixture of two alkaloids with very similar retention times Thus, further VLC separation led to the isolation of two previously unreported alkaloids, camalexin (λmax 214, 274, 318nm) and a structurally related compound (λmax 218, 296, 324nm).

Camalexin, obtained as white crystals, has a molecular formula of $C_{10}H_8N_2S$ as determined by high resolution electron impact mass spectrometry (EI-MS) and verified by chemical ionization MS (CI-MS). Its IR spectrum shows NH absorption, while its UV spectrum suggests the presence of the indole nucleus as well as another chromophoric system. The ^1H NMR spectrum of camalexin shows signals for the indolic (exchangeable) N-H coupled to an aromatic hydrogen at δ7.32. Six additional aromatic hydrogens are observed, four of which (δ8.68 H-4, 7.26 H-5, 7.18 H-6, 6.93 H-7) are assigned to the benzenoid ring of an indole nucleus by spin decoupling experiments. Nuclear Overhauser enhancement (nOe) experiments cause the enhancement of signals at δ7.32 (H-2) and δ6.93 (H-7) upon irradiation of the N-H signal indicating the presence of a 3-substituted indole nucleus. This partial structure is further indicated for camalexin by its ^{13}C nmr spectrum[9]. The isolated spin system (δ7.71, d J=3Hz coupled to δ6.61, d J=3Hz) in the ^1H NMR along with ^{13}C NMR data suggest that the substituent at C-3 is a thiazole, indicating that camalexin possesses either structure **5** or **6**.



5 R=H
8 R=OCH_3



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Both **5** and **6** are biogenetically reasonable: structure **5** through condensation of indole-3-carboxaldehyde with cysteine, cyclization, and decarboxylation; structure **6** by oxidative cyclization of an ylid derived from brassinin (1) Interestingly, compound **6**, which is not known as a natural product, has been synthesized[10] in a search for

compounds with a biological profile similar to that of the well known synthetic fungicide, thiabendazole (7)[11]. However, the spectral data for 6 is not reported. Of the two possible structures for camalexin, 5 is favored since the coupling constants of the isolated spin system observed in its ^{1}H nmr spectrum are more consistent with those of a 2-substituted thiazole ($J_{4,5}$=3-4 Hz)[12]. Camalexin crystallizes as colorless plates from methanol. A single crystal X-ray crystallographic study allowed differentiation between these structures and established that camalexin indeed possesses structure 5. Figure 1 is a computer generated perspective drawing of the final X-ray model of camalexin (5)*.

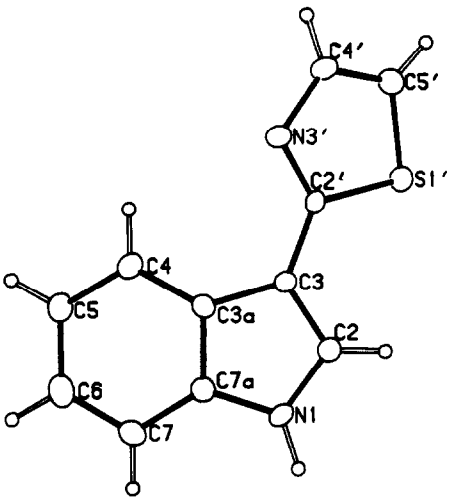
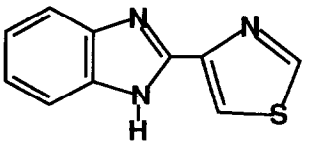


Fig. 1. A computer generated perspective drawing of camalexin.

The second alkaloid ($C_{11}H_{10}N_2SO$) isolated from the fluorescent bioactive component differs from camalexin (5) by a methoxyl group (δ_H3.88 s, 3H; δ_C55.7, q) as indicated by comparison of the ^{1}H and ^{13}C NMR spectra of the two compounds. The methoxyl group in methoxycamalexin is located at C-6 since nOe studies show that irradiation of the N-H signal enhances the signal at δ6.89 (a multiplet with two small couplings, H-7) as well as that at δ7.77 (d, J=2.5Hz, H-2). Thus methoxycamalexin possesses structure 8 In the initial chemical studies of the phytoalexins produced by *C. sativa*, the major component consisted of only compound 8. Subsequent extracts of the leaf material revealed the major component is a mixture of compounds 5 and 8, with compound 8 comprising about 15% of the mixture. We are currently investigating the reasons for the differences in phytoalexin production.

* The X-ray crystallographic study was carried out by Dr. B.D. Santarsiero at the Structure Determination Laboratory, Department of Chemistry, University of Alberta. Inquiries regarding the crystallographic results should be directed to the above address quoting report SDL:WAA9001.



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It is interesting to note that camalexin and methoxycamalexin appear to be the first reported naturally occuring antifungal compounds which contain a 2-substituted thiazole. Thiabendazole (**7**), a synthetic systemic fungicide first developed as an anthelmintic agent and antiinfective agent in animals[13], has been used extensively to control a variety of plant diseases since the early 70's. Thiabendazole is a 4-substituted thiazole[11]. This compound, thought to be an antimitotic agent, has been shown to reduce germ tube elongation of *Penicillium atrovenetum* G Smith[14] and to cause malformation of germ tubes of *Venturia inaequalis* (Cke.) Wint. apud Thuem.[15]. Such phenomena have also been observed with germinating conidia of *A. brassicae* in the presence of camalexin (**5**) and methoxycamalexin (**8**)[16].

Experimental

General. Mass spectra were recorded on an AEI model MS-50 mass spectrometer. The formulas of all peaks reported were determined by high resolution measurements. IR spectra were recorded on a Nicolet 7199F T interferometer. ^{1}H and ^{13}C NMR spectra were determined on Bruker WH-300, WM-360, or WH-400 spectrometers with either Aspect 2000 or 3000 computer systems. Melting points were recorded on a Fisher-Johns melting point apparatus and are uncorrected. Skellysolve B refers to Skelly Oil Co. light petroleum, b p 62-70°C. All chromatography solvents were distilled prior to use.

Isolation and separation of camalexin and methoxycamalexin. Camelina sativa leaves (1000) were inoculated with conidia or with mycelium (grown in V8 juice broth then blended) of *A. brassicae* and maintained in moist Petri dishes as previously described[5]. After 2 - 5 days, the inoculated leaves were extracted with aqueous methanol (H_2O:CH_3OH 3:7). The combined washings (12 L) were concentrated *in vacuo* to 250 mL. A bioautographic technique[5,6] employing *Cladosporium* sp. and silica gel TLC plates indicated the presence of two phytoalexins (R_f 0.43, 0.33; $CHCl_3$-CH_3OH 49:1) in the extract and this technique, in addition to visualization with Dragendorff's reagent, was employed to assist with a bioassay directed fractionation. The aqueous extract was applied to an Extube (300 mL capacity, Analytichem), allowed to distribute over the specially modified diatomaceous earth during 5 minutes, then the organic metabolites were eluted with $CHCl_3$ (700 mL). The $CHCl_3$ eluant was concentrated (10 mL) and extracted with 5% HCl (3 x 20 mL) for 10 minutes. The aqueous acidic extract was cooled and basified to pH 10 with NaOH pellets, then applied to an Extube (100 mL capacity). After 5 minutes the organic bases were eluted with $CHCl_3$ (400 mL). This extract was concentrated (3 mL), combined with two other extracts obtained in the same manner (total 9 mL), then subjected to VLC[7,8] over TLC grade silica gel 60 G (6 cm ID x 4 cm). The compounds of interest were carefully eluted with $CHCl_3$ (100 mL fractions) while monitoring the chromatography with UV light (254 nm, major component fluoresces) in the dark. A small amount of the minor alkaloid component was isolated and further studies on it are underway in these laboratories. Fractions 7 - 9 containing the major alkaloid component contaminated with a small amount of other

material were combined and concentrated (3 mL), then again subjected to VLC over a silica gel 60 HR (extra pure, E. Merck #7744, 60Á; 4 cm ID x 3 cm) eluting with $CHCl_3$ (40 mL fractions). Fractions containing the major component show a single spot on TLC (silica gel; R_f 0.44; $CHCl_3$:CH_3OH 20:1) with phosphomolybdic acid and heating. However, during the chromatography it was observed that color development with phosphomolybdic acid (no heating), UV maxima, and HPLC analysis (C-18 reverse phase (Alltech), 250 x 4.6 mm; eluant: CH_3OH:H_2O 1:1; chart speed 1 cm/min) were different between early and late fractions, establishing that the major component is a mixture of the two alkaloids with retention times of 9.89 (**8**) and 10.11 (**5**) minutes. Careful VLC fractionation with collection of small fractions led to the isolation of compounds **5** and **8**. Bioassay using *Cladosporium* sp.[5,6] shows that each of camalexin (**5**) and methoxycamalexin (**8**) are antifungal agents.

Camalexin (**5**): Isolated as colorless crystals (methanol), m.p. 134-137°C from benzene; UV λmax (CH_3OH) 214 (ε=22,200), 274 (ε=7,900), 318 (ε=13,800) nm; FTIR (KBr) 3432, 3154, 3084, 2921, 1621, 1562,1484, 1456, 1347, 1306, 1295, 1244, 1209, 1146, 1134, 1128, 1098, 1054, 919, 759, 738, 706 cm^{-1}; HRMS (EI, probe, 150°C) m/z 200.0403 meas, 200.0437 calc for $C_{11}H_8N_2S$ (100), 172 ($C_{10}H_6NS$, 3), 155 ($C_{10}H_7N_2$, 3), 142 ($C_9H_6N_2$, 26), 128 (C_9H_6N, 2), 115 (C_8H_5N, 12); (CI NH_3) m/z 203 (13), 202 (36), 201 (100, M^++1), 200 (59, M^+); ^1H NMR (360 MHz, $CDCl_3$) δ9.00 (br s, D_2O exchangeable, N-H), 8.16 (m, H-4, {7.31, 7.19, 7.22}), 7.77 (d, J=3Hz, H-4', {7.17}), 7.74 (d, J=2.5 Hz, H-2, {NH}), 7.31 (m, H-7, {8.16, 7.22}), 7.22 (m, H-6), 7.19 (m, H-5), 7.17 (d, J=3Hz, H-5'); (Benzene-d_6) 8.68 (d, H=8Hz, H-5), 7.71 (d, J=3Hz, H-4', {7.17}), 7.32 (d, J=2.5 Hz, H-2), 7.26 (dt, J=1,7Hz, H-5), 7.18 (dt, J=1,7Hz, H-6), 6.93 (d, J=8Hz, H-7), 6.61 (d, J=3Hz, H-5'); ^{13}C NMR (75.5 MHz, $CDCl_3$, APT) δ163.4 (s, C-2'), 142.2 (d, C-2), 136.5(s, C-7a), 124.8 (s, C-3a), 124.6 (d, C-4'), 123.2 (d, C-5), 121.5 (d, C-6), 120.5 (d, C-4), 116.0 (d, C-5'), 112.2 (s, C-3), 111.7 (d, C-7).

Methoxycamalexin (**8**): Isolated as an oil, crystallizes from methanol-Skellysolve B , m.p.157-159°C; UV λmax (CH_3OH) 218 (ε=21,700), 296 (ε=11,500), 324 (ε=11,250) nm; FTIR ($CHCl_3$ cast) 3270, 3120, 2954, 2925, 2854, 1629, 1540, 1456, 1318, 1300, 12489, 1200, 1167, 1111, 1094, 1084, 1050 cm^{-1}; HRMS (EI, probe, 150°C) 230.0515 meas, 230.0511 calc for $C_{12}H_{10}N_2OS$ (100), 215 ($C_{11}H_7N_2OS$, 73), 200 ($C_{11}H_8N_2S$, 15), 187 ($C_{10}H_7N_2S$, 22), 157 ($C_9H_5N_2O$, 7), 129 ($C_8H_5N_2$, 12), 102 (C_7H_4N, 5); (CI-NH_3) 231.64 (M^++H, 13), 230.64 (M+, 100), 200 (M^+-30, 54), 195 (14), 123 (48); ^1H NMR (360 MHz, $CDCl_3$) δ8.32 (br s, D_2O exchangeable, N-H {7.77}),8.13 (d, J=8Hz, H-4 {6.95}), 7.82 (d, J=3Hz, H-4'{7.23}), 7.77 (d, J=2.5Hz, H-5' {8.32}), 7.23 (d, J=3Hz, H-5' {7.82}), 6.95 (m, H-5 {8.13}), 6.89 (m, H-7 {8.13}), 3.88 (s, OCH_3); ^{13}C NMR ($CDCl_3$), 163.2 (s, C-2'), 157.2 (s, C-6), 142.6 (d, C-2), 137.2 (s, C-7a), 123.2 (d, C-4'), 121.4 (d, C-4), 119.1 (s, C-3a), 115.8 (d, C-5'), 112.6 (s, C-3), 111.3 (d, C-5), 94.9 (d, C-7), 55.7 (q, OCH_3).

Acknowledgements

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