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Phytoalexins produced in the leaves of *Capsella bursa-pastoris* (shepherd's purse)

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Three phytoalexins, camalexin (C₁₁H₈N₂S), 6-methoxycamalexin (C₁₂H₁₀N₂SO), and N-methylcamalexin (C₁₂H₁₀N₂S), were isolated from shepherd's purse (*Capsella bursa-pastoris*) challenged by *Alternaria brassicae*. N-methylcamalexin has not been reported previously. Phytoalexin elicitation in shepherd's purse is associated with its resistance to *A. brassicae*, a pathogen which causes an important disease of cruciferous crops in many parts of the world.

[Phytoalexines produites dans les feuilles du *Capsella bursa-pastoris* (bourse-à-pasteur)]

Trois phytoalexines, à savoir la camalexine (C₁₁H₈N₂S), la 6-méthoxycamalexine (C₁₂H₁₀N₂SO) et la N-méthylcamalexine (C₁₂H₁₀N₂S) furent isolées de la bourse-à-pasteur (*Capsella bursa-pastoris*) après une exposition à l'*Alternaria brassicae*. La N-méthylcamalexine n'avait pas été rapportée précédemment. La production de phytoalexines chez la bourse-à-pasteur est reliée à sa résistance à l'*A. brassicae*, un agent pathogène causant, chez les crucifères cultivées, une maladie importante à plusieurs endroits dans le monde.

INTRODUCTION

Alternaria brassicae causes the economically important black spot disease of Brassica crops in many parts of the world (Tewari 1991). Resistance in false flax [*Camelina sativa* (L.) Crantz] to *Alternaria brassicae* (Berk.) Sacc. is associated with the production of phytoalexins (Conn *et al.* 1988; Jejelowo *et al.* 1991). These phytoalexins were subsequently identified as camalexin and 6-methoxycamalexin (Browne *et al.* 1991). Camalexin was also later reported from *Arabidopsis thaliana* (L.) Heynh. challenged with *Pseudomonas*

syringae pv. *syringae* van Hall (Tsuji *et al.* 1992). Because of recent interest in these compounds (Ayer *et al.* 1992; Gross 1993; Pedras *et al.* 1997; Tsuji *et al.* 1993), we report on the isolation of these compounds, and a new related compound, from shepherd's purse [*Capsella bursa-pastoris* (L.) Medic.] challenged with *A. brassicae*. *Capsella bursa-pastoris* is a common weed belonging to the family Brassicaceae and is highly resistant to *A. brassicae* and *A. brassicicola* Groves & Skolko (Conn *et al.* 1988; Sigareva and Earle 1997; Tewari and Conn 1993). It is also cold tolerant, short life-cycled, and resistant to

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flea beetles (Bonfils *et al.* 1991; Sigareva and Earle 1997). All these are useful traits required for genetic improvement of Brassica crops and some recent studies have focussed on this area (Bonfils *et al.* 1991; Sigareva and Earle 1997).

MATERIALS AND METHODS

General

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Infrared spectra (IR) were obtained using a Nicolet 750 FTIR spectrophotometer. Electron impact mass spectra (EIMS) were obtained using a Kratos AEI MS50 high resolution mass spectrometer. Ultraviolet (UV) spectra were recorded on a Hewlett-Packard 8450A diode array spectrometer. ^1H nuclear magnetic resonance spectra ($^1\text{H-NMR}$) were obtained using the following spectrometers: Bruker WH-360 (360 MHz), Bruker AM-400 (400 MHz), and Varian Unity-500 (500 MHz). Coupling constants are reported within ± 0.5 Hz. ^{13}C nuclear magnetic resonance spectra were obtained on a Bruker AM-300 (75 MHz) and Bruker AM-400 (100.6 MHz). Carbon-13 multiplicities were determined using spin echo J-modulated experiments (APT or Attached Proton Test). Nuclear Overhauser Enhancement (NOE) experiments were determined in the difference mode in which a control spectrum was computer-subtracted from the irradiated spectrum after Fourier Transformation. Positive enhancements appear as signals possessing opposite phase with respect to the irradiated signal. Samples for NOE measurements were deoxygenated with argon for 10-20 min prior to use. Homonuclear decoupling experiments were performed using the Bruker DISNMR software package. High Performance Medium Pressure Liquid Chromatography (HPMPLC) was carried out on ACE Glass Inc. Michel-Miller equipment using a solvent pump from Fluid Metering Inc. model RP-SY. Compounds were detected using the ISCO V4 wavelength absorbance detector, and fractions were collected with the ISCO fraction collector model 820. High

Pressure Liquid Chromatography (HPLC) analyses were performed on a Waters 600E System Controller equipped with a 490E Programmable multiwavelength U.V. detector, and M730 Data Module.

Plant material

Plants were grown for 6-8 wk in a greenhouse at approximately 18/12°C (day/night) and a light intensity 400-600 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Plants were also collected from fields in Central Alberta. All plants used, including the siliques thereon, were lush green.

Fungal strains

Stock cultures of the fungus *A. brassicae* (University of Alberta Micro Fungus Herbarium culture #UAMH 7476) were stored on slants in tubes on V-8 juice agar (200 mL filtered V-8 juice, 0.05 g rose bengal, 3 g CaCO_3 , 20 g agar, 800 mL H_2O) at 4°C. *Cladosporium* sp. (to be used in bioassay) was stored on tube slants on malt extract agar (30 g malt extract, 15 g agar, 1 L H_2O) at 4°C. *Alternaria brassicae* was aseptically transferred from slants to Petri dishes containing V-8 juice agar. The cultures were allowed to grow in the dark at room temperature for 10-12 d and then stored at 4°C. Inoculum was prepared by washing the conidia from the plate with 10 mL of sterile, distilled water. The resulting aqueous suspension ($10\ 000\text{-}20\ 000$ spores mL^{-1}) was used to inoculate liquid media. *Alternaria brassicae* was grown in V-8 juice medium (200 mL filtered V-8 juice, 0.75 g CaCO_3 , 800 mL H_2O). The cultures (500 mL liquid medium in 1 L-Fernback flask) were kept in the dark at room temperature under still-culture conditions. The fungus was harvested after 12 d. The mycelium was separated from the broth by gravity filtration through cheese cloth. This mycelium was blended with sterile distilled water to obtain a suspension which was used in phytoalexin-elicitation experiments as follows.

Phytoalexin elicitation

Detached plant material (1 kg leaves and in some cases whole plants without roots but with flowers and siliques) was dipped into a mycelial suspension

and then placed in a humid chamber in plastic trays lined with moist paper towels and covered with clear polyethylene sheets. The control was dipped in distilled water and also placed in a humid chamber. The plant material was brought from the greenhouse and the field, and incubated on a laboratory bench at room temperature under continuous white fluorescent light for 5 d. Phytoalexins were extracted from the plant material before senescence occurred, and saprophytic growth of *A. brassicae* took place.

Multiwell method

The isolation of active metabolites was bioassay-directed as described by Trifonov *et al.* (1992). The sample was sterilized by filtration through a 0.22 μm nylon membrane. A 200 μL sample of the fraction to be bioassayed was applied directly to the well of the multiwell plate (6-well, flat bottom, Falcon 3046) using a micropipette. The solvent was evaporated in a sterile fume-hood for 3 h. The inoculum was obtained from the growing edge of a colony of *Cladosporium* sp. in malt agar Petri dishes. After 4 d of growth at room temperature, a plug (7 mm diam) cut from this culture was placed in each well of the multiwell plate. Three replicates for each fraction and the solvent (control) were evaluated in each multiwell plate. This procedure was repeated for all fractions which showed the same R_f value at each chromatographic step. Inhibition in the radial growth of *Cladosporium* sp. relative to the control was regarded as a positive result.

Isolation of active metabolites

Five d after inoculation, the leaves were macerated in a Waring blender with methanol-water (7:3, 12 L). The filtered extract was concentrated under reduced pressure to 500 mL, water (100 mL) was added and the solution was extracted with ethyl acetate (3 x 500 mL). This extract was washed with 5% aqueous HCl, then made basic with aqueous NH_4OH and extracted with dichloromethane. Removal of the solvent left a viscous oil (0.6 g). Flash chromatography of the crude extract afforded a mixture of three compounds ($R_f=0.35-$

0.50, 2% methanol in dichloromethane). HPMLC (RP-18, 20-40 μm) using 60% aqueous methanol on this mixture afforded camalexin and 6-methoxycamalexin. HPLC (μ Bondpak™ C-18, 7.8 x 300 mm, 125Å) using 50% aqueous methanol afforded N-methylcamalexin.

The camalexin (1) and 6-methoxycamalexin (2), as shown on Fig. 1, were identified by comparison (^1H NMR, IR, UV) with authentic samples (available in our laboratories, Browne *et al.* 1991).

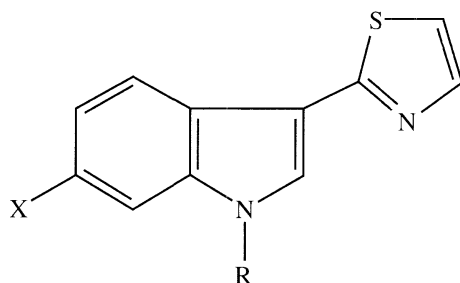
N-methylcamalexin. ^1H NMR (CDCl_3): δ 7.74 (s, H-2), 8.16 (dd, J = 8, 1 Hz, H-4), 7.30 (m, H-5), 7.35 (m, H-6), 7.45 (dd, J = 8, 1.5 Hz, H-7), 7.8 (d, J = 2.5, H-4'), 7.20 (d, J = 2.5 Hz, H-5'), 3.86 (s, 3H, N-CH₃); Calculated for $\text{C}_{12}\text{H}_{10}\text{N}_2\text{S}$, 214.2844; found 214.2840 amu.

RESULTS AND DISCUSSION

In bioassay, the activity was found to reside in the basic material. Flash chromatography of the basic fraction followed by medium pressure liquid chromatography on reversed phase silica gel provided the two major components. HPLC of the residues provided small amounts of a third component.

The major component ($R_f=0.35$) had a molecular formula of $\text{C}_{11}\text{H}_9\text{N}_2\text{S}$ as determined by EIMS. Absorption at 3430 cm^{-1} in the IR spectrum suggested the presence of an NH group. This observation is consistent with the UV spectrum ($\lambda_{\text{max}} = 214, 274, 318$) for an indole moiety. The ^1H NMR showed seven aromatic signals at δ 8.68, 7.26, 7.18, 6.93, 7.77, and 7.17 and a broad signal at δ 9.0 for the indolic (exchangeable) hydrogen.

In NOE experiments, the presence of a 3-substituted indole moiety was detected. The hydrogens at δ 7.3 (H2) and δ 6.9 (H7) exhibited enhancement upon irradiation of the N-H signal (δ 8.6). Homodecoupling experiments on the spin system H4'-H5' in the thiazole moiety showed the appropriate coupling constants (J_{4,5} = 3 Hz) (Jackman and Sternhell 1969). Therefore, the presence of a 2-substituted thiazole was



- (1) R = X = H
 (2) R = H, X = OCH₃
 (3) R = CH₃, X = H

Figure 1. Structures of camalexin (1), 6-methoxycamalexin (2), and N-methylcamalexin (3).

substantiated. Comparison IR, ¹H NMR, ¹³C NMR spectra of this compound with an authentic sample of camalexin (1) confirmed the identity (Fig. 1).

The second compound showed a molecular formula C₁₂H₁₀N₂SO, suggesting an additional methoxy group. Indeed, its ¹H NMR showed a singlet at δ3.88 and ¹³C NMR showed a new signal at δ55.7. Absorption at higher wavelength in the UV spectrum was consistent with this observation. The relative position of a methoxy group on the aromatic ring was determined by NOE experiments. The hydrogens at δ6.8 and δ7.7 exhibited enhancement upon irradiation of the signal at δ8.6 (N-H). In addition, signals at δ6.94 and δ6.87 showed 2.8 and 3.2% NOE, respectively, upon irradiation of the signal at δ3.88 (methoxy group). Comparisons with an authentic sample of 6-methoxycamalexin (2) completed the identification (Fig. 1).

Further analysis of the crude organic extract by HPLC (RP-C18, 50% aqueous methanol) showed the presence of a third compound. The molecular formula C₁₂H₁₀N₂S indicated the addition of one methyl group to camalexin. The main difference observed in the ¹H-NMR of this compound, compared with the two other camalexins, was the presence of an N-methyl signal at δ3.86. The lack of an O-methyl signal and the

similarity of the remainder of the ¹H NMR spectrum with that of camalexin (1), suggested that this minor metabolite possesses the structure (3) (N-methylcamalexin) (Fig. 1). This compound has not been reported previously.

None of the compounds 1, 2, or 3 were isolated from plant material in the control experiment. These compounds satisfy the definition of phytoalexins (Paxton 1981) in *C. bursa-pastoris* as they are antimicrobial and are accumulated in the plant after challenge by the pathogen. Also, they resemble other phytoalexins from plants of the family Brassicaceae in containing an indole moiety and sulphur (Gross 1993).

Some research has been done on the role of camalexin in the host-defense systems of *C. sativa* and *A. thaliana* (Conn *et al.* 1988, 1994; Jejelowo *et al.* 1991; Rogers *et al.* 1996; Tsuji *et al.* 1992). Camalexin is the major phytoalexin in *C. bursa-pastoris* and is associated with high degrees of resistance to *A. brassicae* and *A. brassicicola* (Conn *et al.* 1988; Jejelowo *et al.* 1991; Sigareva and Earle 1997). It has also been shown to be toxic to *A. brassicae* (Conn 1991; J.P. Tewari and P. Kutschy, unpublished data), *A. solani* (Jejelowo 1995), and *Rhizoctonia solani* Kühn (Conn *et al.* 1994) in *in vitro* studies. EC₅₀ for inhibition of conidial germtube growth of *A. brassicae* is approximately

6 $\mu\text{g mL}^{-1}$ and the MIC (minimum inhibitory concentration) for complete inhibition of germ tube growth is 80 $\mu\text{g mL}^{-1}$ (Jejelowo *et al.* 1991). Low yields of phytoalexins, such as 6-methoxycamalexin and N-methylcamalexin have so far not permitted such studies.

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