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Components from the phytotoxic extract of *Alternaria brassicicola*, a black spot pathogen of canola

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Abstract

Six new fusicoccane-like diterpenoids, brassicicene A to F, have been isolated from the liquid culture filtrates of the canola pathogen *Alternaria brassicicola*. Their structures have been established using a broad range of spectral evidence including 2D NMR spectroscopic experiments. The isolation of this class of diterpenoids from the genus Alternaria has not been previously reported.

Keywords: *Alternaria brassicicola*; Cotylenins; Fusicoccins; Diterpenoid; Canola pathogens; *Brassica napus*; Cruciferae

1. Introduction

Alternaria brassicicola (Schw.) Wiltshire is a fungus which causes dark leaf spot in winter oilseed rape (*Brassica napus* L. *ssp. oleifera* Metzg.). Its infection of canola results in premature pod shattering, seed loss and infection of the seeds which results in reduction of crop yields and quality of oil (Hodgkins & MacDonald, 1986). *A. brassicicola* was responsible for the 20–50% yield loss seen in infected fields of winter rape in Germany (Daebeler, Riedel, & Riedel, 1986). The disease manifests itself visibly on the leaves where it appears as black, sooty, velvety lesions with yellow halos around them (chlorotic zones) (Verma & Saharan, 1994). The pathogen attacks most parts of the plant and it is thought to induce its chlorotic effect by the excretion of phytotoxins.

In the literature there are very few reports that address the secondary metabolite production of *A. brassicicola*. Investigation of the antibiotic complex produced by *A. brassicicola* have lead to the isolation of brassicicolin A (acylated D-mannitol derivative) from the culture filtrate (Gloer, Poch, Short, & McCloskey, 1988). A cytokinin containing fraction prepared from a culture filtrate has been implicated in *A. brassicicola*'s pathogenesis (Suri & Mandahar, 1984). Lastly, depudecin, a C_{11} compound containing two epoxide groups, has been isolated from the culture broth of *A. brassicicola* RF-328 and has been shown to exhibit cell differentiation — modulating activity (Matsumoto et al., 1992). In this paper we report the bioassay directed isolation and identification of six potentially phytotoxic fusicoccane-like compounds, brassicicene A–F, from the culture filtrate of *A. brassicicola*.

2. Results and discussion

The fungus was grown in liquid still culture for 14 days on a medium containing 20% V-8 juice. The mycelial mat was separated from the culture broth by filtration, dried and extracted with ethyl acetate. The culture broth was extracted with ethyl acetate after it had been concentrated to 1/20 of its original volume. Only the broth extract exhibited activity when both crude extracts were tested for chlorotic activity using the detached canola leaf-spot bioassay (Ayer & Pena-Rodriguez, 1987). The active extract was separated

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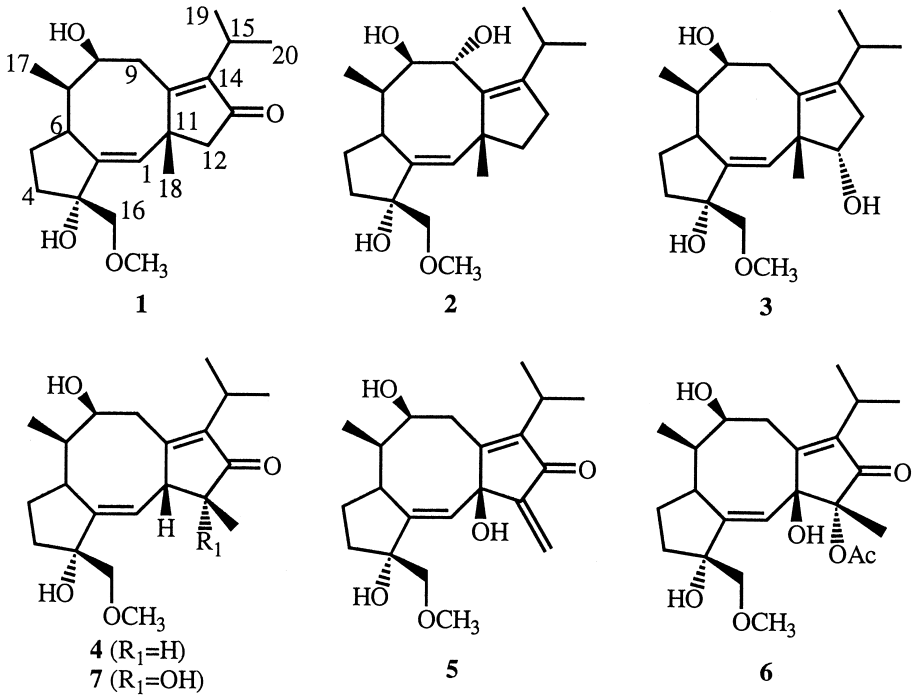

Scheme 1

into five fractions using Sephadex LH-20 column chromatography. Fractions 3 and 4 demonstrated the strongest chlorosis-inducing ability. Semi-purification of the major components of these active extracts was accomplished by Sephadex LH-20 chromatography. Purification of the six title compounds, brassicicene A through F, was accomplished by reverse-phase semi-preparative HPLC.

Table 1
^{1}H NMR spectral data for compounds **1**, **3**–**7** in CD_3OD (δ ppm) (coupling constants ( ) in Hz)

H	1	3	4	5	6	7
1	5.74 d (2.5)	5.48 d (2.5)	6.09 dd (2.0, 10.0)	6.00 d (2.0)	5.89 d (1.5)	6.13 dd (1.8, 10.8)
4	1.45 m	1.31 m	1.53 m	1.41 m	1.55 m	1.52 ddd (3.4, 6.5, 13.0)
	1.97 m	1.92 m	1.87 m	1.98 m	1.88 dd (6.5, 13.0)	1.85 m
5	1.39 m, 1.97 m	1.26 m, 1.92 m	1.44 m, 1.97 m	1.47 m, 1.75 m	1.47 m, 1.97 m	1.44 m, 1.95 m
6	2.71 br d (6.3)	2.80 dt (2.5, 7.5)	2.80 br d (7.5)	2.70 br d (6.5)	2.76 br d (7.0)	2.84 br d (7.5)
7	1.89 m	1.71 dq (4.0, 7.0)	2.03 dq (3.5, 7.5)	1.82 br q (7.3)	1.97 m	2.04 m
8	3.93 dt (3.5, 10.5)	3.77 dt (4.0, 11.5)	4.17 ddd (3.5, 5.5, 11.5)	4.04 ddd (3.0, 6.3, 10.3)	4.20 ddd (4.0, 5.5, 12.0)	4.16 ddd (3.9, 5.7, 11.7)
9	2.56 m	1.94 m	2.45 dd (11.5, 13.5)	2.60 dd (6.3, 12.3)	2.65 dd (12.0, 16.0)	2.93 m
		2.26 m	2.91 m	3.07 dd (10.8, 12.3)	3.01 dd (4.0, 16.0)	2.44 dd (11.7, 14.3)
11			3.35 dd (5.5, 10.0)			3.09 d (10.8)
12	2.27 dd (1.0, 18.5)	3.63 dd (7.0, 9.0)	3.13 m 2.47 d (18.5)			
13		1.87 m, 2.21 dd (7.0, 14.2)				
15	2.85 h (7.0)	2.79 h (7.0)	2.92 h (7.0)	2.99 h (7.0)	2.98 h (7.0)	2.90 h (6.5)
16	3.27 d (10.0)	3.11 d (10.0)	3.38 d (10.0)	3.26 d (10.0)	3.39 d (10.0)	3.38 d (10.0)
	3.43 d (10.0)	3.33 d (10.0)	3.48 d (10.0)	3.32 d (10.0)	3.46 d (10.0)	3.46 d (10.0)
17	0.90 d (7.0)	0.74 d (7.0)	1.00 d (7.5)	0.88 d (7.5)	0.94 d (7.0)	0.99 d (7.0)
18	1.32 s	1.05 s	1.25 d (7.5)	5.21 s	1.69[b] s	1.57 s
19	1.25[a] d (7.0)	0.94[a] d (7.0)	1.32[a] d (7.0)	1.31[a] d (7.0)	1.11[a] d (7.0)	1.20[a] d (6.5)
20	1.17[a] d (7.0)	0.91[a] d (7.0)	1.15[a] d (7.0)	1.20[a] d (7.0)	1.30[a] d (7.0)	1.33[a] d (6.5)
21	3.38 s	3.30 s	3.37 s	3.35 s	3.37 s	3.36 s
Ac					1.89[b] s	

[a] Assignments in the same column may be reversed.
[b] Assignments in the same column may be reversed.

Table 2
^{13}C NMR spectral data for compounds **1** and **3**–**7** in CD_3OD (δ ppm)

C	1	3	4	5	6	7
1	134.4	129.6	127.7	132.2	133.5	127.3
2	141.9	144.7	147.7	146.6	150.0	151.3
3	83.0	83.7	84.1	83.0	84.1	84.0
4	36.4	36.1	36.6	36.3	36.4	36.5
5	32.8	33.1	38.0	35.0	37.5	38.0
6	42.0	42.5	47.9	45.3	46.3	47.3
7	45.9	45.9	50.9	48.3	50.2	50.5
8	76.2	76.9	75.5	74.5	74.8	75.6
9	31.3	29.1	30.6	30.3	32.5	29.0
10	175.6	133.6	176.7	161.1	171.6	172.4
11	48.2	54.5	58.8	82.1	90.6[a]	65.4[a]
12	53.2	83.3	44.8	153.4	89.1[a]	81.2[a]
13	209.3	35.2	207.4	202.3	202.2	200.0
14	146.5	143.4	151.2	147.3	150.0	149.8
15	27.0	28.3	28.9	27.2	29.5	29.0
16	78.1	78.2	79.5	78.4	79.2	79.3
17	8.5	8.6	12.3	10.3	12.0	12.9
18	28.2	25.0	13.0	103.2	15.1[b]	21.5
19	20.2	21.0	19.0	18.8	21.9	21.7
20	20.0	20.7	22.0	21.1	17.9	18.9
21	59.7	59.6	59.6	58.9	59.6	59.6
22					167.9	
23					21.0[b]	

[a] Assignments in the same column may be reversed.
[b] Assignments in the same column may be reversed.

Brassicicene A (**1**) was a white solid which had a $[M]^+$ peak corresponding to $C_{21}H_{32}O_4$. The ^1H NMR spectrum displayed two methyl proton doublets at δ 1.25 and 1.17 coupled to a one proton heptet at δ 2.85 (gCOSY) thus indicating the presence of an isopropyl group. The presence of an α,β unsaturated ketone was evident from the absorption at 1679 cm^{-1} in the IR spectrum and by the two quaternary olefinic (δ 175.6 and 146.5) and one ketone (δ 209.3) resonance in ^{13}C NMR. This absorption for a cyclopentenone ring seemed lower than what was anticipated but inspection of the literature revealed that a range from 1680–1710 cm^{-1} could be expected (Herz & Lakshmikantham, 1965; Itah, Nozoe, Tsuda, & Okuda, 1967; Stuart & Barrett, 1969). The IR spectrum also showed a hydroxyl band at 3391 cm^{-1}. The ^1H NMR displayed an olefinic proton at δ 5.74, a methoxy group at δ 3.38 (s), and one additional methyl group (CH_3–CH) at δ 0.90 (d). The skeleton structure of brassicicene A was elucidated from an INADEQUATE spectrum which displayed all of the carbon–carbon correlations in the molecule except that for C1 to C2 (Scheme 1). Many of the ^1H and ^{13}C shifts, Tables 1 and 2 respectively, were assigned and verified using APT, HMQC and gCOSY spectra.

A search of the literature revealed that brassicicene A was very closely related to the fusicoccin and cotylenin diterpenoids (Muromtsev et al., 1994). These compounds contain the same dicyclopenta[a,b]cyclooctane skeleton as **1** and have been described in fungi, algae, liverworts and insects. The presence of a methoxymethylene group was supported by the characteristic fragmentation of this moiety seen in the EIMS at 303 $[M\text{-}45]^+$. Its placement at C3 was based on comparison to the NMR data of cotylenol (**2**). By analogy with the fusicoccanes, it is assumed that the absolute stereochemistry is as shown (Muromtsev et al., 1994).

An $[M + Na]^+$ peak corresponding to $C_{21}H_{32}O_4Na$ was obtained for brassicicene B (**3**) from the HRESIMS analysis. The IR spectrum of **3** contained a hydroxyl band at 3352 cm^{-1}. The IR absorption for an α,β unsaturated ketone and a ketone resonance in the ^{13}C NMR spectrum were not present. The ^{13}C NMR contained one methine (δ 129.6) and three quaternary olefinic carbons (δ 144.7, 133.6 and 143.4). As in brassicicene A, the ^1H NMR and gCOSY spectra confirmed the presence of the C-14 isopropyl group and the C-7 methyl group. The methoxymethylene group was verified by the $[M–H_2O–CH_2OCH_3]^+$ peak at 287 in the EIMS spectrum and by the characteristic AB system at δ 3.22 and the singlet at δ 3.37 (OCH_3) in the ^1H NMR spectrum. Assignment of most of the structure and its ^1H and ^{13}C resonances was accomplished using APT, HMQC, gCOSY and HMBC spectra as well as by comparison to **1**.

The assignment of the quaternary methyl group at C11 was aided by the C1 to C11 to C18 correlation seen in the INADEQUATE spectrum. Because of the

size of the sample and time constraints the INADEQUATE spectrum only showed carbon–carbon correlations for a few fragments found in brassicicene B. The remaining methine (δ 83.3) and methylene (δ 35.4) groups were assigned to C12 and C13 respectively based on the following NMR spectroscopic evidence. The magnitude of the chemical shift of the methine carbon indicated that it was hydroxylated. Correlation's between C1 and the methine proton, and H18 and the methine carbon were visible in the HMBC spectrum. No HMBC correlations for the protons or carbons of the methylene group were observed. The hydroxylated methine group was therefore assigned to C12. This assignment was supported by the interactions which were seen between H12 and both the H18 methyl protons and H13 methylene protons, in the NOESY spectrum. The C13 protons exhibited a nOe to the C12 protons but not to the C18 methyl protons. The C12 hydroxyl group was assigned the α stereochemistry because the NOESY spectrum showed an nOe between the β C18 methyl protons and C12 proton. In addition to this evidence, there are many examples of this C12 α-hydroxy stereochemistry reported in the literature (Ballio et al., 1972; Ballio, 1979).

Brassicicene C (**4**) was isolated as a white solid and gave a $[M]^+$ of 348.2297 (corresponding to $C_{21}H_{32}O_4$) in its HREIMS spectrum. Its structure differs from **1** only in the regiochemistry of the C18 methyl group which is now proposed to have undergone a migration from C11 to C12. Evidence of the hydroxyl (3405 cm^{-1}) and α,β unsaturated ketone (1685 cm^{-1}) groups was present in the IR spectrum. The presence of the C14 isopropyl group and the C3 methoxy–methylene group was confirmed using the same analysis used for brassicicene A. Comparison of the ^1H and ^{13}C NMRs of **1** and **4** revealed the following differences: (1) a C18 methyl group at δ 1.25 appearing as a doublet ($J = 7.5$ Hz) instead of the singlet seen in **1**; (2) a C1 olefinic proton at δ 6.09 that was now present as a doublet of doublets ($J = 2.0$, 10.0 Hz); (3) two methine protons appearing at δ 3.13 (m) and 3.35 (dd).

Inspection of the gCOSY spectrum confirmed the expected allylic coupling (2.0 Hz) between H1 (δ 6.09) and H6 (δ 2.80) which had been observed in the other brassicicene NMR spectra. The vicinal coupling (10.0 Hz) of H1 to a methine proton at δ 3.35 led to the placement of this single H at C11. The remaining methine hydrogen at δ 3.13 was assigned to C12 because of its coupling (7.5 Hz) to the methyl doublet at δ 1.25. Assignment of these two methine protons adjacent to each other was confirmed by correlation's in the gCOSY spectrum.

When the C17 methyl group was irradiated, in a cycle nOe experiment, an enhancement was seen in the C18 methyl group signal thus confirming the assigned stereochemistry at C12. Until now no fusicoccane-like compounds containing a C12 methyl group have been reported in the literature.

The HREIMS spectrum of brassicicene D (**5**) showed a $[M]^+$ at 362.2091 which corresponded to $C_{21}H_{30}O_5$ and its structure therefore contained one more double bond equivalent than the previously discussed brassicicenes. Comparison of the ^1H and ^{13}C NMR spectra of brassicicene D (**5**) with that of the other brassicicene compounds revealed the loss of the C18 methyl group, the presence of an olefinic methylene group at δ 103.2, and an quaternary olefinic carbon at δ 153.4. In the ^1H NMR the olefinic methylene group appeared as a 2H singlet (δ 5.21) when determined in CD_3OD but this changed to two 1H singlets (δ 5.17 and 5.29) when the spectrum of **5** was obtained in $CDCl_3$. An external double bond was placed at C12–C18 because of C12's chemical shift of δ 161.1 and C18's chemical shift of δ 103.2 which agreed with what was expected for a terminal olefinic methylene group (Silverstein, Bassler, & Morrill, 1991). A hydroxyl group was placed at C11 because of the magnitude of this quaternary carbons shift of δ 82.1. It was tentatively assigned the β-stereochemistry but requires verification by X-ray crystallography when more sample is available.

In the HREIMS spectrum an $[M–HCO_2CH_3]^+$ of 362.2098, corresponding to $C_{21}H_{30}O_5$, was observed for brassicicene E (**6**). The molecular formula of $C_{23}H_{34}O_7$ for **6** was confirmed by the $[M + NH_4]^+$ at 440.6 in the CIMS, and the $[M]^+$ at 422 in the EIMS. The loss of m/z of 60 from the molecular ion in the HREIMS spectrum implied the presence of an acetate group. The IR spectrum contained absorptions indicative of hydroxyl (3390 cm^{-1}), acetate carbonyl (1733 cm^{-1}) and an α,β unsaturated ketone (1712 cm^{-1}).

When the ^1H and ^{13}C NMR spectra were compared to those of the other brassicicenes, it was concluded that **6** differed only in its functionalization at C11 and C12. APT, gCOSY and HMQC spectra were also employed in the assignment of most of the chemical shifts given in Tables 1 and 2. The methyl group at δ 21.0, which appeared as a singlet at δ 1.89 in the ^1H NMR spectrum, and the quaternary carbon at δ 167.9 were assigned to the acetate moiety. This acetate, a methyl group appearing as a singlet in the ^1H NMR at δ 1.69, one hydroxyl group and two oxygenated quaternary carbons (δ 90.6 and 89.1) were the last elements to be included in the structure of **6**.

The two quaternary carbons were assigned to C11 and C12 but a definitive assignment could not be made even with the aid of the HMBC spectrum. The chemical shift of these two carbons indicated that each was oxygenated so the option of attaching the acetate and the hydroxyl group to just one of these two car-

bons was eliminated. This resulted in the attachment of the C18 methyl group to C12. The proposed placement of the hydroxyl and acetate groups given in structure **6** is based on the previously isolated brassicicenes but as yet requires further verification. This would be accomplished by X-ray crystallography when more sample can be isolated.

The last of the title compounds, brassicicene F (**7**), differed from brassicicene C only by one hydroxyl group. An $[M–H_2O]^+$ at 346.2142 in the HREIMS and a $[M + NH_4]^+$ at 382.4 confirmed the molecular formula to be $C_{21}H_{32}O_5$. Analysis of the IR, MS and ^1H and ^{13}C NMR data confirmed the presence, as in **4**, of the isopropyl group, the methoxymethylene group and the α,β unsaturated ketone. The methine proton at δ 3.09 was assigned to C11 because of its coupling ($J = 10.8$ Hz) to H1, which was visible in the gCOSY spectrum of **7**. The remaining methyl group, a singlet at δ 1.57, was attached to C12. The positioning of a hydroxyl group at C12 explained this carbons quaternary chemical shift of δ 81.2. A cycle nOe experiment confirmed the stereochemistry of the C18 methyl group as its signal was enhanced when the C17 methyl group was irradiated.

Fusicoccane-like diterpenoids have not been previously reported in the fungal genus *Alternaria*. Their occurrence was unexpected because previous work done in our lab on *A. brassicae*, another black spot pathogen of canola, resulted in the isolation of cyclodepsipeptides as the phytotoxic principles (Ayer & Pena-Rodriguez, 1987). Inspection of the ^1H NMR spectra throughout the isolation process did not indicate the presence of these polypeptide compounds in the ethyl acetate extract of the culture broth. The phytotoxicity of the brassicicenes have not been suitably evaluated due to the small amounts of compounds that were isolated. No significant antimicrobial activity was exhibited by these compounds, when tested using the disc diffusion bioassay, against *Aspergillus fumigatus* UAMH 2978, *Bacillus subtilis*, *Candida albicans* ATCC 18804, *Microsporum canis* UAMH 4174, *Petromyces albertensis* UAMH 2976, and *Trichophyton mentagrophytes* UAMH 5888.

3. Experimental

3.1. General experimental procedures

Water for fungal culturing was distilled in an all glass apparatus prior to use. Silica gel 270–400 Mesh was used for flash chromatography. Optical rotations were determined on a Perkin Elmer model 241 polarimeter set on the sodium D line (589 nm). Compounds were dissolved in spectroscopic grade methanol. Solution and crystal IR spectra were recorded on a Nicolet Magna 750 microscope FT interferometer. HREIMS were recorded on an A.E.I. MS-50 mass spectrometer coupled to a DS-50 data system. HRESIMS (high resolution electrospray ionization mass spectrometry) spectra were obtained using a Micromass ZabSpec Hybrid Sector-TOF spectrometer using a 1% solution of acetic acid in H_2O:MeOH as a liquid carrier. CIMS spectra were obtained using a Modify A.E.I. Kratos MS-9 mass spectrometer equipped with a CI source run in reagent ammonia gas. All MS data are recorded as m/z. All NMR spectra were obtained on a Varian Unity 500 (500 MHz) spectrometer and were obtained in methanol-d_4. The Inadequate spectra were obtained using a ^{13}C NanoNMR probe.

Semipreparative HPLC was performed on a Waters 600E System Controller equipped with a 490E programmable multiwavelength UV detector and a M730 Data Module. The column used was a reverse-phase (C18) μBondapak™ PrepPak Cartridge (10 μm particle size, 25 cm × 100 mm) installed in a 25 × 10 RCM™ cartridge holder.

3.2. Culturing of A. brassicicola

The strain of *A. brassicicola* used in this study was isolated by Petrie and Jasalavich in May 1991 from the seeds of *Brassica oleracea ssp. capitata* 'Houston Evergreen' (cabbage) from British Columbia, Canada. *A. brassicicola* strain UAMH 7474 was deposited with the University of Alberta Microfungus Collection. Stock cultures were retrieved from liquid nitrogen storage and plated on V-8 juice agar plates (200 mL V-8 juice, 0.05 g rose bengal, 3 g $CaCO_3$, 20 g agar, 800 mL H_2O) which were then incubated at room temperature. After 10–14 days of growth the inoculum culture was prepared by adding 10 mL of sterile water to each plate. The culture was then suspended in the water by scratching the submerged surface of the plate. Aliquots of this suspension were then aseptically transferred into Fernbach flasks containing 1 L of sterilized, liquid V-8 juice media (200 mL V-8 juice, 0.75 g $CaCO_3$, 800 mL H_2O). The fungus was incubated at room temperature in the dark and harvested after 14 days of growth.

3.3. Preparation of crude mycelial and broth extracts

The black mycelial mat was separated from the broth by filtration through a large Buchner funnel and allowed to dry in the fumehood (approximately 2 days). It was then extracted with EtOAc for 24 h using a soxhlet extractor. A crude mycelium extract was obtained after removal of the organic solvent under reduced pressure.

The filtered broth was concentrated under reduced

pressure (bath temperature 35°C) to 1/20 of its original volume. The concentrated broth was then extracted five times with 500 mL of EtOAc. The organic extracts were combined, dried over $NaSO_4$ and concentrated under reduced pressure to yield a brown crude broth extract.

3.4. Detached leaf-spot bioassay

The phytotoxicity of the crude extracts were evaluated using the young leaves of greenhouse-grown canola (*B. napus* cv. Westar). The leaves were obtained from canola plants that were grown in a greenhouse in the Agriculture and Food Sciences Department at the University of Alberta. The surface of each leaf was gently scratched in four, well separated, sites with the tip of a glass pasteur pipette. A 10 μL aliquot of a 5% methanol solution of the crude extract was applied to each scratched site. Extracts were tested in triplicate and a methanol control was included in each run. The methanol in the applied sample was evaporated off using a gentle stream of nitrogen. The canola leaf was then maintained at room temperature in a closed petri dish containing a moistened piece of filter paper. Chlorosis was observed within 48 h (Ayer & Pena-Rodriguez, 1987; Bains, Tewari, & Ayer, 1993).

3.5. Fractionation of the broth extract and isolation of six diterpenoids

The EtOAc extract (1.7 g) of the broth was divided into 5 fractions by CC on Sephadex LH-20 using a CH_2Cl_2: Me_2CO gradient. These fractions were bioassayed for phytotoxicity using the detached leaf spot bioassay at concentrations equivalent to the 5% crude extracts assayed initially. Fractions 3 and 4 elicited the most chlorosis. Further fractionation of these active fractions was accomplished using CC on Sephadex LH-20 with isocratic elution with CH_2Cl_2. Isolation of the six title compounds from the generated fractions was accomplished using semipreparative HPLC. This involved the isocratic elution of a Radial-Pak reverse-phase column with 35% CH_3CN in H_2O. The column was eluted at a flow rate of 6 mL/min and the eluent was monitored by a UV detector set a 206 nm. Melting points are not reported for the following brassicicenes as the compounds decomposed before melting.

3.6. Brassicicene A (**1**)

(24 mg from 23 L of culture); IR (crystal) ν_{max} cm^{-1}: 3391 (O–H), 2900 (C–H aliphatic), 1679 (α,β unsaturated ketone), 1619 (C=C); HREIMS, found. $[M]^+$ 348.2303; $C_{21}H_{32}O_4$ requires 348.2301; ^1H and ^{13}C NMR, Tables 1 and 2 respectively; EIMS *m/z* (rel. int.): 348 $[M]^+$ (2), 330 $[M–H_2O]^+$ (3), 303 $[M–CH_2–O–CH_3]^+$ (84), 285 $[M–CH_2–O–CH_3–H_2O]^+$ (100), 243 $[M–CH_2–O–CH_3–H_2O–CH_2{=}CHCH_3]^+$ (47); $[\alpha]_D^{23}$ +44.4° (*c* 0.1350).

3.7. Brassicicene B (**3**)

(9.1 mg from 28 L of culture); IR(crystal) ν_{max} cm^{-1}: 3352 (O–H), 2910 (C–H aliphatic); HRESIMS, found. $[M + Na]^+$ 373.2355; $C_{21}H_{34}O_4Na$ requires 373.2355; ^1H and ^{13}C NMR, Tables 1 and 2 respectively; EIMS *m/z* (rel. int.): 332 $[M–H_2O]^+$ (100), 287 $[M–H_2O–CH_2–O–CH_3]^+$ (12), 229 $[M–H_2O–CH_2–O–CH_3–C_3H_6O]^+$; $[\alpha]_D^{23}$ −9.1° (MeOH; *c* 0.2636).

3.8. Brassicicene C (**4**)

(4.6 mg from 14 L of culture); IR ($CHCl_3$ cast) ν_{max} cm^{-1}: 3405 (O–H), 2960 (C–H aliphatic), 1685 (α,β unsaturated ketone); HREIMS, found. $[M]^+$ 348.2289; $C_{21}H_{32}O_4$ requires 348.2301; ^1H and ^{13}C NMR, Tables 1 and 2 respectively; EIMS *m/z* (rel. int.): 348 $[M]^+$ (2), 303 $[M–CH_2–O–CH_3]^+$ (50), 301 (77), 285 $[M–CH_2–O–CH_3–H_2O]^+$ (49), 243 $[M–CH_2–O–CH_3–H_2O–CH_2{=}CHCH_3]^+$ (37); $[\alpha]_D^{23}$ −66.6° (*c* 0.0150).

3.9. Brassicicene D (**5**)

(4 mg from 23 L of culture); IR (crystal) ν_{max} cm^{-1}: 3400 (O–H), 2890 (C–H aliphatic), 1708 (α,β unsaturated ketone), 1645 (C=C); HREIMS, found. $[M]^+$ 362.2091; $C_{21}H_{30}O_5$ requires 362.2093; ^1H and ^{13}C NMR, Tables 1 and 2 respectively; EIMS *m/z* (rel. int.): 362 $[M]^+$ (17), 344 $[M–H_2O]^+$ (9), 317 $[M–CH_2–O–CH_3]^+$ (18), 299 $[M–CH_2–O–CH_3–H_2O]^+$ (45), 259 $[M–CH_2–O–CH_3–C_3H_6O]^+$ (30); $[\alpha]_D^{23}$ −156.0° (*c* 0.1250).

3.10. Brassicicene E (**6**)

(1.5 mg from 15 L of culture); IR (MeOH cast) ν_{max} cm^{-1}: 3390 (O–H), 2936 (C–H aliphatic), 1733 (C=O acetate), 1712 (α,β unsaturated ketone); HREIMS, found. $[M–HCO_2CH_3]^+$ 362.2098; $C_{21}H_{30}O_5$ requires 362.2093; ^1H and ^{13}C NMR, Tables 1 and 2 respectively; CIMS (NH_3) *m/z* (rel. int.): 440.6 $[M + NH_4]^+$ (4), EIMS 422 $[M]^+$ (2), 404 $[M–H_2O]^+$ (1), 362 $[M–CH_3COOH]^+$ (37), 359 $[M–H_2O–CH_2–O–CH_3]^+$ (13), 344 $[M–CH_3COOH–H_2O]^+$ (30); $[\alpha]_D^{23}$ −53.3° (*c* 0.0600).

3.11. Brassicicene F (**7**)

(1.8 mg from 15 L of culture); IR(crystal) ν_{max} cm^{-1}: 3400 (O–H), 2920 (C–H aliphatic), 1690 (α,β unsaturated ketone); HREIMS, found. $[M–H_2O]^+$

346.2142; $C_{21}H_{30}O_4$ requires 346.2144; ^1H and ^{13}C NMR, Tables 1 and 2 respectively; CIMS (NH_3) m/z (rel. int.): 382.4 $[M + NH_4]^+$, EIMS 346 $[M–H_2O]^+$ (13), 319 $[M–CH_2OCH_3]^+$ (15), 301 $[M–H_2O–CH_2–O–CH_3]^+$ (100), 243 $[M–H_2O–CH_2–O–CH_3–C_3H_6O]^+$ (43); $[\alpha]_D^{23}$ −81.7° (*c* 0.0600).

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