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C-METHYL FLAVONOLS FROM THE FUNGUS *COLLETOTRICHUM DEMATIUM* f.sp. *EPILOBII*

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Key Word Index—fungal metabolites; C-methyl flavonols; *Colletotrichum dematium* f.sp. *epilobii*; *Epilobium angustifolium* L. ssp. *angustifolium*; 5,4′-dihydroxy-3,7,8-trimethoxy-6-C-methylflavone; 5,4′-dihydroxy-3,6,7-trimethoxy-8-C-methylflavone.

Abstract—Two C-6 and C-8 methyl flavonols were isolated from a fungus and identified for the first time. The fungus *Colletotrichum dematium* f.sp. *epilobii* is a disease of fireweed, *Epilobium angustifolium* L. ssp. *angustifolium* (family Onagraceae); both disease and plant are indigenous to Canada. *C. dematium* secretes several metabolites when grown in liquid culture. Two novel natural product compounds: 5,4′-dihydroxy-3,7,8-trimethoxy-6-C-methylflavone and 5,4′-dihydroxy-3,6,7-trimethoxy-8-C-methylflavone were isolated from ethyl acetate extracts of liquid culture filtrates. Structures were confirmed on the basis of UV ^{1}H NMR, ^{13}C NMR spectroscopy, as well as mass spectroscopic techniques and represent the first report of C-methylflavonols produced by fungi.

INTRODUCTION

Fungi have a number of characteristics that make them suitable biological weed control agents including the facts that many occur naturally on plant hosts, which may be targets of agricultural and silvicultural weed control programs, and that they can be mass-produced via liquid fermentation [1]. The development of various commercial bioherbicides in North America (e.g. COLLEGO®, DEVINE®, BIOMAL®) [2] has led to increasing scientific research on various fungal species, particularly members of the *Colletotrichum* genus, as potential biological weed control agents. One such organism that has been investigated as a biological weed control agent is *Colletotrichum dematium* f.sp. *epilobii*, an indigenous pathogen of fireweed, *Epilobium angustifolium* L. ssp. *angustifolium* (family Onagraceae) [3]. *Colletotrichum dematium* f.sp. *epilobii* causes large necrotic lesions on the leaves and stem of infected fireweed and appears to be specific to *E. angustifolium* and possibly a few other members of the genus [3].

Secondary metabolites produced by plants and microorganisms represent a wide range of chemical structures and mechanisms of action that have potential utility in biorational pest management strategies, as well as possible pharmaceutical applications. Studies on bioactive natural product compounds produced by plant pathogens are also critical to the full understanding of host-pathogen interactions. Public concern over artificial chemical methods of pest control has stimulated increased interest in natural products as possible sources of new ecologically friendly pest management strategies. Biorational and biological pest (weed and insect) management strategies aim to keep weed and insect populations at levels low enough that they can co-exist with the crop species without affecting crop growth and development.

Fungi produce a variety of compounds including peptides, terpenoids, macrolides and phenolics [4]. Gloeosporone, the spore germination self-inhibitor (1*S*, 6*R*, 12*R*)-1-hydroxy-6-pentyl-5,15-dioxabicylo [10.2.1]pentadecan-4,13-dione, was isolated from *Colletotrichum gloeosporioides* by Meyer *et al.* [5, 6]. Kimura *et al.* [7], Lunnon and MacMillan [8], Suzuki *et al.* [9], Kosuge *et al.* [10] and Gohabara *et al.* [11] have all reported the isolation of several orssellinaldehydes possessing a monoprenyl or diprenyl side chain at C-3 together with or without a chlorine atom at C-5 (colletotrichin *A*, *B* and *C*, and colletochlorin *D*) and an α-pyrone compound (colletopyrone) from *Colletotrichum nicotianae*. Other compounds, *meso*- and D-(−)-butane-2,3-diol, col-

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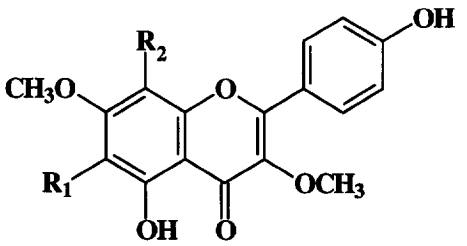

1 $R_1 = CH_3$; $R_2 = OCH_3$
2 $R_1 = OCH_3$; $R_2 = CH_3$

letruncoic acid methyl ester and 2-hydroxymethylhexa-2,4-dienol, were isolated from *Colletotrichum truncatum* [12]. In addition, *Epilobium* species have been shown to contain many types of flavonoids [13].

The purpose of the present research was to isolate, identify and elucidate the chemical structures of various metabolites secreted by *C. dematium* f.sp. *epilobii* in culture in relation to the possible role of its metabolites in host–pathogen interaction. Ongoing studies are directed at final isolation and identification of the phytotoxic principals. This paper reports for the first time the presence of two rare 6- and 8-C-methylflavonols in *Colletotrichum dematium* f.sp. *epilobii* and fungi in general.

RESULTS AND DISCUSSION

Initial results of bioassay-guided fractionation of *Colletotrichum dematium* f.sp. *epilobii* culture filtrates demonstrated a high degree of antimicrobial and phytotoxic activity for the ethyl acetate fraction, which ultimately resulted in isolation of the two compounds described below. However, subsequent bioassay using a *Lemna gibba* G3 microplate assay demonstrated that neither of the isolated compounds was phytotoxic at dose levels up to 1.5 μg ml^{-1}. We report here the identification of two novel C-methylflavonols, which represents the first time that this class of flavonoid has been isolated from a fungus. Current continuing studies are directed at final isolation and identification of the phytotoxic principals. No trace of flavonoids were detected by PC, TLC or HPLC analysis of an ethyl acetate extract of the uninoculated sterile culture broth, nor in the ethyl acetate and ethanol extracts of PDA and PDB from the bottle.

When these extracts were chromatographed at concentrations of PDA and PDB equivalent to that in the culture medium, no spots were visible under UV before or after spraying with three spray reagents and no peaks appeared at 280 or 350 nm in HPLC traces. By contrast, at least five spots or HPLC peaks appeared when the fungal culture extract was examined. We therefore conclude that no flavonoid precursors were available in the sterile medium in the amounts sufficient to account for the appearance of compounds **1** and **2**.

Flavonoid compounds are found throughout the plant kingdom, but they are infrequent in fungi. Bird and Marshall have reported the isolation of chlorflavonin (3′-chloro-5,2′-dihydroxy-3,7,8-trimethoxyflavone) from cultures of *Aspergillus candidus* [14]. The isoflavonoids are a subclass of the flavonoids metabolized by many microorganisms; modified structures have been identified when specific compounds have been added to the microbial cultures. Microbial metabolites of natural isoflavonoids and modified structures thereof have been reviewed by Dewick [15].

In a recent review by Wollenweber [16], a total of 89 C-methylflavonoids were reported in plants, but none in fungi. The majority of these compounds have some exclusive character, i.e. each occurs in a single plant species. Few C-methylflavonoids have been found in more than one plant species and rarely in species from different families.

The UV spectrum of compounds **1** and **2** in methanol and the shapes and positions of bands I and II after the addition of $AlCl_3$ and $AlCl_3$ + HCl suggested that this was a 3-methoxyflavone with a free hydroxyl at C-5, monosubstituted on the B-ring and substituted at C-6 and C-8, respectively [17]. Lack of a free 7-hydroxyl in both compounds was suggested by the absence of shift of band II after the addition of sodium acetate [18] and the absence of band III after the addition of sodium methoxide [19]. Furthermore, the latter reagent revealed the presence of a free hydroxyl at C-4′ in both cases. Accordingly, the two compounds appeared to be 6,7,8-trisubstituted derivatives of 3-methoxykaempferol.

Compound **1**, $C_{19}H_{18}O_7$ $[M]^+$ exhibited a molecular peak at *m/z* 358 (48.2) in accordance with a flavonol containing two hydroxyl and four methyl groups. ^{1}H-NMR spectroscopic data indicated a monosubstituted B ring [δ 7.98 (2H) and δ 6.98 (2H)]. As the ^{1}H-NMR spectrum showed one signal at δ 2.05 (3H) and three resonances, respectively, at δ 3.79 (3H) 3.87 (3H) and 3.95 (3H), the compound is substituted by one aryl methyl group and three methoxy groups. The presence of a peak at *m/z* 211 (2.5%) indicated that the *A* ring bears two methoxyl and one methyl groups [20]. The fragment ion at *m/z* $[M-Me]^+$ 343 (100%) and the absence of any $[M-H_2O]^+$ ions in the mass spectrum of **1** are in agreement with the ^{1}H NMR NOE and ^{1}H, ^{13}C HMQC NMR discrimination between the substitution at C-6 and C-8. ^{1}H NMR NOE experiments showed an enhancement of an H-2′/H-6 doublet during irradiation of the protons on two of the methoxy substituents. Molecular models show 3-OMe and 8-OMe to be in close proximity to the aforementioned aromatic protons. Irradiation of the 6-Me protons produced an enhancement of only the 7-OMe signal. In 6-OMe compounds, $[M]^+$ is usually more important than $[M-Me]^+$, often appearing as the base peak, whereas in those with a methoxy at C-8, the

base peak is due to $[M-Me]^+$ [21]. Furthermore, 6-OMe compounds exhibit $[M-H_2O]^+$ greater than 10%, while in 8-OMe isomers, this ion is lower than 10%. Compound **1** is therefore 5,4′-dihydroxy-3,7,8-trimethoxy-6-C-methylflavone.

A small amount of compound **2** was isolated from the ethyl acetate extract. With a molecular peak at *m/z* 358 (100%), the second compound appeared to be the 8-C-methyl-6-O-methyl isomer of **1**. Its mass spectrum suggested that it is a flavonol with two hydroxyl and four methyl groups ($C_{19}H_{18}O_7$). In the mass spectrum, the presence of a peak at *m/z* 211 (2.2%) indicates that the A ring bears two methoxyl and one methyl groups [20]. The fragment ion at *m/z* $[M-Me]^+$ 343 (55.4%) and the presence of the $[M-H_2O]^+$ 340 (11) ion in the mass spectrum of **2** agree with the UV data. Acid hydrolysis of compound **2** gave rise to two compounds (a mixture of the two isomers **1** and **2**). Both co-chromatographed with compounds **1** and **2**. Both 6- and 8-C-flavonoids interconvert (Wessely–Moser rearrangement) to a mixture of the 6- and 8-isomers under standard acid hydrolysis conditions [18]. Compound **2** is therefore provisionally assigned the structure 5,4′-dihydroxy-3,6,7-trimethoxy-8-C-methylflavone.

Comparison of the UV, ^1H NMR and MS data of compound **1** and **2** with the published data of 5,4′-dihydroxy-3,7-dimethoxy-6,8-di-C-methylflavone by Rasamoelisendra *et al.* [22] shows that compound **1** and **2** are different and new natural product compounds.

EXPERIMENTAL

Growth of Colletotrichum dematium *and extraction of metabolites.* Isolate cultures of *C. dematium* f.sp. *epilobii* (ATCC no. 2081) were maintained as small mycelial sections in 10% glycerol in cryovials at −80°. Cultures were initiated from sections thawed at 30° on 3.4% potato dextrose agar (PDA) and grown for 4 days at 25° in the dark. Three 7 mm diameter pieces, cut from the edge of the agar culture, were transferred into 50 ml of liquid 3.4% potato dextrose broth (PDB) in 250 ml flasks. The inoculated flasks were incubated at 25° in the dark on an orbital shaker at 200 rpm for 5 days, then their contents were triturated in a Sorvall omnimixer at a setting of 5 for 1 min in an ice bath. Twenty-five ml of the fungal slurry so obtained were added to a 15 l fermentor flask containing 4 l of PDB. After incubation for 5 days under the same conditions described above, the culture was filtered through miracloth under vacuum. Purity of the culture was checked by plating samples on nutrient agar and PDA prior to filtration. The filtrate was maintained at 2° until the sterility of the culture was confirmed, then extracted with ethyl acetate (3 × 4 l). The combined EtOAc extracts were concd *in vacuo*. An EtOAc extract of 4 l of sterile uninoculated culture broth was similarly prepd. The concd EtOAc extract was subjected to PC, TLC or HPLC analysis, as described below.

Examination of the sterile medium for presence of flavonoids. Since potato tubers from which PDA and PDB are made contain flavonoids [23], it was considered essential to examine the PDB culture medium for their possible presence. An EtOAc extract of 4 l of sterile uninoculated culture broth was prepd in the manner as the fungal filtrate. In addition, PDA powder (100 g) and PDB liquid (100 ml) were each extracted with (1) 2 × 500 ml of EtOAc and (2) sequentially with 500 ml each of EtOH, EtOH–H_2O (7:3) and EtOH–H_2O (1:1). The resulting four extracts were concd and all five extracts subjected to 2D PC, both silica gel and cellulose TLC and HPLC with two solvent systems, as described below. In addition to 5% $AlCl_3$ and Naturstoffreagenz A chromatograms were sprayed with vanillin–HCl and sodium borohydride ($NaBH_4$–HCl) for detection of catechins and flavanones.

Isolation of metabolites. Concd extracts were chromatographed in two dimensions on Whatman no. 1 chromatography paper (PC) and cellulose (TLC) using BAW (*n*-butanol–acetic acid–water, 4:1:5, upper phase) and acetic acid–water (15:85). The extracts were also subjected to unidimensional TLC on silica gel with CH_2Cl_2–$(CH_3)_2CO$ (3:1). The developed chromatograms were sprayed with 5% $AlCl_3$ solution and Naturstoffreagenz A to detect flavonoid compounds. EtOAc extracts of culture broth were fractionated on a flash column packed with 50 g of RP-silica gel G60 (BDH) using CH_2Cl_2 as the eluent, followed by increasing concs of Me_2CO in CH_2Cl_2. A small portion of each fr. was removed for bioassay with *Cladosporium cucumerinum* on TLC [24] and *Lemna gibba* G3 microplate [25]. Further fractionation was carried out on a PVP (Sigma) column using the following solvent systems: (i) CH_2Cl_2–EtOH–MeCOEt–Me_2CO (1:1:1:1); (ii) EtOH–MeCOEt–Me_2CO–H_2O (1:1:1:1); and (iii) EtOH–H_2O (1:1). Purification was achieved on a Chemco low-prep pump, model 9 1-M-8R (with 6-port valve, max. 80 ml min^{-1}) and Bio-Rad S-X beads using C_6H_{14}–CH_2Cl_2–EtOAC (1:1:1). Final purification of the compounds was achieved on a Sephadex LH-20 column (1 cm × 50 cm) using MeOH as the eluting solvent [4, 26].

High performance liquid chromatography. (HPLC) EtOAc frs were sepd on a Nova-Pak C18 60A° 4 μm (3.9 × 150 mm) (Waters) reverse phase column using a gradient technique, a Walter Delta Prep 4000, an autoscan photodiode array detector 996 and Millennium 2010 software. Two gradient programs were used at room temp: (i) solvent A = MeOH; B = 5% aq. HCOOH, as in [27], but the flow rate was set at 1.2 ml min^{-1} instead of 3 ml min^{-1}, (ii) solvent A = MeOH–acetonitrile (95:5); B = 5% aq. HCOOH [28]. Two fixed detection wavelengths were used: 280 and 350 nm. Resolved peaks were scanned by the photodiode array detector from 250 to 400

nm. Retention times (min) of the isolated compounds were: (i) **1**, 31.8 and **2**, 30.9 and (ii) **1**, 38.1 and **2**, 37.0.

Identification of the isolated compounds. UV spectra were recorded on a UV-Vis Beckman spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AMX-500 spectrometer, at 500 MHz and 125 MHz, respectively; samples were dissolved in DMSO-d_6 with TMS as an internal standard. Elemental analysis was performed by M-H-W Laboratories, Phoenix, Arizona, USA. Structures of purified compounds were determined according to standard methods: UV spectroscopy; ^{1}H NMR; ^{13}C NMR and mass spectroscopy [29–32].

5,4′-*Dihydroxy*-3,7,8-*trimethoxy*-6-*C*-*methylflavone*. UV λ^{MeOH}: 276, 295 (sh), 333, 367 (sh); +MeONa: 254 (sh), 274, 398 stable; +$AlCl_3$: 284, 309, 366, 417 (sh); +$AlCl_3$–HCl: 286, 307, 355, 419; +NaOAc: 275, 294 (sh), 333, 368 (sh); +NaOAc-H_3BO_3: 275, 294 (sh), 333, 368 (sh). EIMS *m/z* (rel.int.): 358 $[M]^+$ (48.2), 357 $[M-1]^+$ (4.6), 343 $[M-Me]^+$ (100), 329 $[M-HCO]^+$ (1.5), 315 $[M-COMe]^+$ (2.3), 212 $[A+H]^+$ (1.0), 211 $[A]^+$ (2.5), 182 $[A-HCO]^+$ (1.2), 168 $[A-MeCO]^+$ (6.7), 153 $[A-MeCOMe]^+$ (1.5), 118 $[B_1]^+$ (1.7), 121 $[B_2]^+$ (19.2). ^{1}H NMR: DMSO-d_6 500 MHz: δ (ppm/TMS) 12.59 (1H, *s*, HO-5), 7.98 (2H, *d*, $J = 8$ Hz, H-2′ and H-6′), 6.98 (2H, *d*, $J = 8$ Hz, H-3′ and H-5′), 3.95 (3H, *s*, OMe), 3.87 (3H, *s*, OMe), 3.79 (3H, *s*, OMe), 2.05 (3H, *s*, C-Me). ^{13}C NMR (DMSO-d_6): δ 178.4 (C-4), 160.5 (C-4′), 156.6 (C-7), 156.0 (C-2), 152.9 (C-5), 146.6 (C-9), 137.9 (C-3), 132.3 (C-8), 130.1 (C-2′), 120.55 (C-1′), 155.8 (C-3′), 112.2 (C-6), 106.7 (C-10), 61.6 (MeO-7), 60.8 (MeO-3), 59.6 (MeO-8), 7.9 (Me-6). Analysis calculated for $C_{19}H_{18}O_7$: H, 5.24; C, 62.42; Found: H, 5.43; C, 62.59.

5,4′-*Dihydroxy*-3,6,7-*trimethoxy*-8-*C*-*methyflavone*. UV λ^{MeOH}: 278, 298 (sh), 335, 370 (sh); +MeONa: 276, 395 stable; +$AlCl_3$: 287, 305, 370, 415 (sh); +$AlCl_3$-HCl: 289, 303, 358, 418; +NaOAc: 277, 297 (sh), 335 369 (sh); +NaOAc-H_3BO_3: 277, 297 (sh), 335, 369 (sh). EIMS *m/z* (rel.int.): 358 $[M]^+$ (100), 357 $[M-1]^+$ (11), 343 $[M-Me]^+$ (54.4), 340 $[M-H_2O]^+$ (11), 329 $[M-HCO]^+$ (3.9), 315 $[M-COMe]^+$ (2.7), 212 $[A+H]^+$ (0.8), 211 $[A]^+$ (2.2), 182 $[A-HCO]^+$ (1.9), 168 $[A-MeCO]^+$ (7.2), 153 $[A-MeCOMe]^+$ (1.9), 118 $[B_1]^+$ (23.1), 121 $[B_2]^+$ (21.2).

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