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SECONDARY METABOLITES FROM *MYCOSPHAERELLA LIGULICOLA**

GEMMA ASSANTE,† LORENZO CAMARDA, LUCIO MERLINI‡ and GIANLUCA NASINI

Centro C.N.R. per le Sostanze Organiche Naturali, Politecnico, Istituto di Chimica, 20133 Milano, Italy; † Istituto di Patologia Vegetale and ‡ Istituto di Biochimica Generale, Facoltà di Agraria dell'Università, 20133 Milano, Italy

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Key Word Index—*Mycosphaerella;* Ascomycete; secondary metabolites; 3-methylidene-1,4-benzodioxan-2-one; structural determination.

Abstract—(+)-Epoxydon, together with the new (+)-epoxydon monoacetate, 3-methylidene-6-methoxy-1,4-benzodioxan-2-one and 2-(2-hydroxy-5-methoxyphenoxy)-acrylic acid, has been isolated and identified from the mycelium of *Mycosphaerella ligulicola* grown on Sabouraud-maltose 4%-agar.

INTRODUCTION

In the course of screening the fungal genus *Mycosphaerella* [1, 2] for secondary and possibly phytotoxic metabolites, we have investigated *Mycosphaerella ligulicola.* This fungus belongs to the Ascomycetes and is often described as *Ascochyta chrysanthemi*; both species are causal agents of florist's chrysanthemum ray blight giving rise to tissue discoloration of the floret, wilting and necrotic lesions forming above the inoculation site [3]. Over the years the incidence of this disease in the areas of chrysanthemum cultivation has caused severe commercial losses because of the flower infection; many studies are reported on the life cycle [4, 5], biological characteristics [6] and environmental conditions affecting this parasite [7, 8]. Many species of the genus *Ascochyta* produce antibiotics and phytotoxins, but only ascochytine seems to play a role in disease development [9, 10]. Recent papers report that the same symptoms were obtained by treatment of the plant with culture filtrates of this fungus [9, 10].

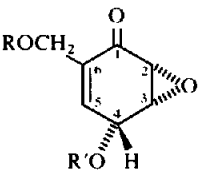
In this paper we report the isolation and structural determination of four metabolites of *M. ligulicola* grown on Sabouraud-maltose–agar.

RESULTS AND DISCUSSION

When *M. ligulicola* was grown on Sabouraud-maltose 4%-agar (SMA) for 2 weeks, two main metabolites, **1** and **2**, were produced; if the fungus was grown for 4 weeks two other metabolites, **3** and **4**, were isolated together with a small quantity of **1** and **2**.

The EtOAc extract of the fungus grown on SMA for 2 weeks was evaporated and chromatographed on preparative TLC plates (silica gel containing 1% KH_2PO_4) to give **1** and **2**. The ^1H NMR spectrum and the optical rotation of **1** corresponded with those of the known compound (+)-epoxydon previously isolated from *Phoma* sp. [11] and from *Phyllosticta* sp. [12]. The phytotoxic [12] and the pharmacological [11] activity of **1** have been studied.

Compound **2** proved to be the novel monoacetyl derivative of (+)-epoxydon at the $-CH_2OH$ group; its spectral data were clear cut and easily interpreted. Treatment of the crude extract of the fungus containing both metabolites **1** and **2** with pyridine/Ac_2O gave almost quantitatively tetra-*O*-acetyl-2,3,5-trihydroxybenzyl alcohol together with a small quantity of (+)-epoxydon diacetate (**2a**); this behaviour of (+)-epoxydon in basic medium has already been reported [11].



1 R = R′ = H
2 R = Ac, R′ = H
2a R = R′ = Ac

Chromatography of the EtOAc extract of the fungus grown for 4 weeks gave the two new metabolites **3** and **4**, which are closely correlated. The ^1H NMR spectrum of **3** showed three protons of a 1,2,4-aromatic system, a OMe group and two protons of a vinyl group. The ^{13}C NMR spectrum gave signals corresponding to ten carbons: five quaternary carbons, including a carbonyl, three aromatic C–H carbons, a carbon of a OMe and finally a carbon corresponding to a $=CH_2$ group. The IR spectrum is in agreement with the presence of a lactone carbonyl (1748 cm^{-1}). High-resolution measurement of the M^+ in the mass spectrum (M^+ = 192), together with the above reported data suggested that the metabolite has structure **3** or alternatively that the aromatic methoxyl is in the 7-position. The choice between these two structures was made on the basis of ^1H NMR spectral analysis of the

* Part X in the series "Secondary Mould Metabolites". For Part IX see Assante, G., Camarda, L. and Nasini, G. (1980) *Gazz. Chim. Ital.* **110**, 629.

compound **7**, obtained by treatment of **3** with diazomethane (to give **5**) and subsequent hydrogenation with Pd on $BaSO_4$. By irradiation of the OMe zone in the ^1H NMR of the compound **7**, the decoupling of all three aromatic protons was observed. Such an effect is consistent with structure **3**, and not with any alternative formulation. Hydrogenation of **3** with Pd on $BaSO_4$ gave **6**. This compound was treated with $LiAlH_4$ in THF to give **8**, which gave the diacetate **9** with pyridine/Ac_2O. This sequence gave further confirmation for structure **3**.

The ^1H NMR spectral signals of **4** were similar to those of **3**, the only difference being the presence of a signal for an OH group at δ 8.65. The IR spectrum of **4** gave characteristic bands at 3290, 2935, 2970 and 1710 cm^{-1} (–COOH). Moreover, treatment of **4** with diazomethane gave **5**, i.e. the same product obtained by treating **3** with the same reagent. All these data showed that **4** is the hydroxy acid corresponding to the lactone **3**. The presence in the side-chain of the enolpyruvic moiety in **3** and in **4** is rather unusual in natural compounds, although such a chain is found, for instance, in chorismic acid, a biogenetic intermediate in the shikimate pathway [13].

We have also examined a strain of *Phoma chrysanthemi* Voglino (DSM Göttingen code 63133) and two strains of *Ascochyta chrysanthemi*, isolated from diseased plants of *Chrysanthemum morifolium*. All these strains appeared to produce both (+)-epoxydon and (+)-epoxydon monoacetate, if cultivated under the same conditions as for *Mycosphaerella ligulicola*.

EXPERIMENTAL

Mps are uncorr. UV spectra were measured in 95% EtOH. NMR spectra were recorded at 90 and 100 MHz, chemical shifts are in ppm (δ), from TMS as internal standard. Column chromatography and TLC were performed with Si gel. Unless otherwise indicated, the purity of products was checked by TLC, NMR and MS and deemed sufficient for the purposes of structural elucidation.

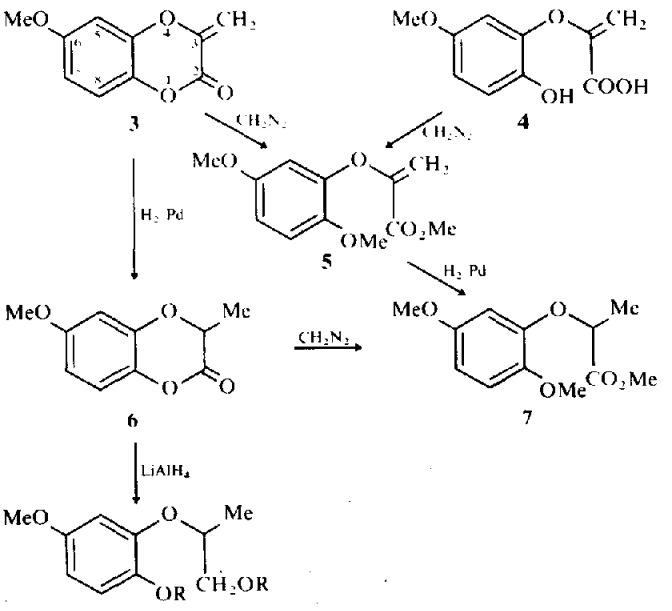
Isolation and purification of metabolites. A strain of *M. ligulicola* (CBS.367.67) obtained from Centraalbureau voor Schimmelcultures, Baarn, grown on Sabouraud-maltose 4%-agar in Roux flasks was extracted twice with EtOAc after 2 weeks' growth at room temp. The extract was dried (Na_2SO_4) and evapd to give a brown mixture of crude metabolites. The mixture was chromatographed by prep. TLC on Si gel containing 1% of KH_2PO_4 using hexane–EtOAc (1:1) and purified further by prep. TLC using $CHCl_3$–MeOH (19:1) as eluant. Two main metabolites, **1** (182 mg) and **2** (30 mg), were isolated from 22 flasks. By growing the fungus under the same conditions for 4 weeks, two other metabolites, **3** (30 mg) and **4** (5 mg), were isolated using similar methods, together with a small amount of **1** and **2**.

(+)-*Epoxydon* (**1**). Oil, $[\alpha]_D^{23°}$ + 91.9° (*c* 0.123, EtOH). ^1H NMR (DMSO, 90 MHz): δ 3.42 (1H, *d*, *J* = 5 Hz, H-2), 3.76 (1H, *m*, H-3), 4.03 (2H, –CH_2–OH), 4.70 (1H, *m*, H-4), 4.97 (1H, OH), 5.78 (1H, *d*, *J* = 5 Hz, OH), 6.42 (1H, *m*, H-5).

(+)-*Epoxydon monoacetate* (**2**). Needles, mp 60–62°. $[\alpha]_D^{23°}$ + 90.4° (*c* 0.125, EtOH). MS *m/z*: 198, 166, 156, 138, 127. IR ν_{max}^{nujol} cm^{-1}: 3430 (OH), 1725 (acetate CO), 1670 (conj. CO). ^1H NMR ($CDCl_3$, 90 MHz): δ 2.07 (3H, *s*, COMe), 3.15 (1H, *br s*, OH), 3.52 (1H, *d*, *J* = 3 Hz, H-2), 3.85 (1H, *m*, H-3), 4.72 (3H, *s*, –CH_2–O– and H-4), 6.53 (1H, *m*, H-5).

(+)-*Epoxydon diacetate* (**2a**). Oil. MS *m/z*: 240, 181, 169, 156, 138, 127. IR ν_{max}^{neat} cm^{-1}: 1745 (acetate CO), 1690 (conj. CO). ^1H NMR ($CDCl_3$, 90 MHz): δ 2.08 and 2.20 (6H, *s*, COMe), 3.53 (1H, *d*, *J* = 4 Hz, H-2), 3.88 (1H, *m*, H-3), 4.75 (2H, *s*, –CH_2–O–), 5.82 (1H, *m*, H-4), 6.40 (1H, *m*, H-5).

3-*Methylidene-6-methoxy*-1,4-*benzodioxan*-2-*one* (**3**). Needles, mp 56–57° (hexane–Et_2O). MS *m/z*: 192.0407 ± 0.002 ($C_{10}H_8O_4$ requires 192.0422), 164, 149, 139, 107. UV λ_{max} nm: 286 and 312 sh (ε 5750, 2500). IR ν_{max}^{KBr} cm^{-1}: 1748 (lactone CO). ^1H NMR ($CDCl_3$, 90 MHz): δ 3.76 (3H, *s*, OMe), 5.28 and 5.82 (2H, *d*, *J* = 3 Hz, =CH_2), 6.57 (2H, *m*, H-5 and H-7), 6.97 (1H, *d*,



J = 8 Hz, H-8). ^{13}C NMR ($CDCl_3$): 157.0 (*s*, CO), 143.3, 139.9 and 133.1 (*s*, C-3, C-6, C-4a and C-8a), 117.5, 108.9 and 101.5 (*d*, C-5, C-7 and C-8), 104.8 (*t*, $=CH_2$), 55.7 (*q*, OMe).

2-(2-*Hydroxy-5-methoxyphenoxy*)-*acrylic acid* (**4**). Glassy solid. MS *m/z*: 192 ($M^+ - 18$), 182, 164, 149, 140, 121. UV λ_{max}nm: 291 (ε 3350). IR ν_{max}^{KBr} cm^{-1}: 3290 (OH), 2970, 2935, 1710 (–COOH). ^{1}H NMR (Me_2CO-d_6, 90 MHz): δ 3.72 (3H, *s*, OMe), 4.80 and 5.60 (2H, *d*, J = 3 Hz, $=CH_2$), 6.67 (2H, *m*, H-4 and H-6), 6.92 (1H, *d*, J = 8 Hz, H-3), 8.65 (*br*, OH).

Methyl 2-(2,5-*dimethoxyphenoxy*)-*acrylate* (**5**). **4** (5 mg) dissolved in dry MeOH was treated with CH_2N_2–Et_2O at room temp. After 10 min evapn of the solvent gave (**5**) as a glassy solid. ^{1}H NMR ($CDCl_3$, 90 MHz): δ 3.73, 3.80 and 3.88 (9H, *s*, OMe), 4.72 and 5.58 (2H, *d*, J = 2 Hz, $=CH_2$), 6.70 (2H, *m*, H-4 and H-6), 6.93 (1H, *d*, J = 8 Hz, H-3).

3-*Methyl*-6-*methoxy*-1,4-*benzodioxan*-2-*one* (**6**). **3** (20 mg) in 10 ml EtOAc was hydrogenated in the presence of 10% Pd on $BaSO_4$ at room temp. for 2 hr. Filtration, evapn of solvent and prep. TLC (hexane–EtOAc, 4:1) gave **6** as an oil. MS *m/z*: 194, 166, 151, 139, 121. UV λ_{max}nm: 235, 281, 284 sh and 290 sh (ε 3950, 3600, 3400, 3000). IR ν_{max}^{KBr} cm^{-1}: 1782 (lactone CO). ^{1}H NMR ($CDCl_3$, 90 MHz): δ 1.62 (3H, *d*, J = 6 Hz, Me–CH–), 3.78 (3H, *s*, OMe), 4.60 (1H, *q*, J = 6 Hz, –CH–Me), 6.60 (2H, *m*, H-5 and H-7), 7.0 (1H, *d*, J = 8 Hz, H-8).

Methyl 2-(2,5-*dimethoxyphenoxy*)-*propionate* (**7**). **6** (20 mg) was dissolved in 5 ml MeOH and treated with CH_2N_2–Et_2O for 5 min. Evapn of solvent and prep. TLC (hexane–EtOAc, 1:1) gave **7** as a glassy solid. MS *m/z*: 240, 225, 210, 181, 166, 139, 125. UV λ_{max} nm: 287 (ε 3300). IR ν_{max}^{neat} cm^{-1}: 1755 (ester CO). ^{1}H NMR ($CDCl_3$, 100 MHz): δ 1.62 (3 H, *d*, J = 6 Hz, Me–CH–), 3.73, 3.75 and 3.81 (9 H, *s*, OMe), 4.77 (1 H, *q*, J = 6 Hz, –CH–Me), 6.48 (1 H, *dd*, J = 2 and 8 Hz, H-4), 6.52 (1 H, J = 2 Hz, H-6), 6.83 (1 H, *d*, J = 8 Hz, H-3). The same compound was obtained by hydrogenation of **5** with Pd on $BaSO_4$.

2-(2-*Hydroxy*-5-*methoxyphenoxy*)-*propanol* (**8**). **6** (20 mg) was dissolved in 5 ml dry THF and $LiAlH_4$ added to the soln until no further evolution of H_2 was observed. The mixture was treated with crushed ice–dil. HCl and extracted with EtOAc. Prep. TLC ($CHCl_3$–MeOH, 15:1) gave almost quantitatively **8** as an oil. MS *m/z*: 198, 166, 151, 140, 125. UV λ_{max}nm: 291 (ε 1700). IR ν_{max}^{neat} cm^{-1}: 3380 (OH). ^{1}H NMR ($CDCl_3$, 90 MHz): δ 1.30 (3H, *d*, J = 6 Hz, Me–CH–), 3.72 (3H, *s*, OMe), 3.5–4.5 (3H, –OCH–Me and –OCH$_2$–CH–), 6.48 (1H, *dd*, J = 2 and 8 Hz, H-4), 6.57 (1H, *d*, J = 2 Hz, H-6), 6.85 (1H, *d*, J = 8 Hz, H-3).

2-(2-*Acetoxy*-5-*methoxyphenoxy*)-*propyl acetate* (**9**). **8** (10 mg) was dissolved in 0.5 ml of dry pyridine and treated with Ac_2O (0.5 ml). The mixture was left to stand at room temp. for 1 hr, dissolved in $CHCl_3$ and treated with satd $NaHCO_3$ soln, H_2O, satd $KHSO_4$ soln, H_2O and finally dried (Na_2SO_4). Evapn of the solvent gave **9** as an oil. MS *m/z*: 282, 240, 198, 180, 165, 151, 140, 125. UV λ_{max}nm: 279.5 and 285 sh (ε 2350, 2000). IR ν_{max}^{neat} cm^{-1}: 1768 and 1742 (acetate CO). ^{1}H NMR ($CDCl_3$, 90 MHz): δ 1.28 (3H, *d*, J = 6 Hz, Me–CH–), 2.04 and 2.25 (6H, *s*, COMe), 3.78 (3H, *s*, OMe), 3.9–4.7 (3H, –OCH–Me and –OCH$_2$–CH–), 6.47 (1H, *dd*, J = 2 and 8 Hz, H-4), 6.58 (1H, *d*, J = 2 Hz, H-6), 6.94 (1H, *d*, J = 8 Hz, H-3).

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