# Metabolites from Endophytes of the Medicinal Plant *Erythrina crista-galli*

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*Erythrina crista-galli* (Fabaceae) is used in Argentinean ethnopharmacology as anti-inflammatory medication, narcotic, desinfectant, and for the treatment of wounds. The common name of the tree is "ceibo" or coral tree. The dominating endophytes in *E. crista-galli* all belong to the genus *Phomopsis* as identified by microscopic features and the analysis of their ITS sequences. To investigate a possible contribution of *Phomopsis* spp. to the metabolites found in the plant, twelve different isolates were cultivated in different media. Besides several new metabolites a number of known compounds were detected: mellein, nectriapyrone, 4-hydroxymellein, scytalone, tyrosol, clavatol, mevinic acid, and mevalonolactone.

*Key words: Erythrina crista-galli*, Endophytic *Phomopsis*, Metabolites

## Introduction

Due its North-South extension in the South American continent, the Argentine Republic has a rich biodiversity. There exist ethnopharmacological data on some 1,800 endemic plants, some still being used in folk medicine. However, most of the plants are still unexplored, and there is scarce information about their chemical composition.

It is assumed that all higher plants are associated with endophytic fungi (Hawksworth, 1991; Stone *et al.*, 2000). These fungi form symptomless infections within the plant tissue at some time of their life cycle and can be isolated after surface sterilization (Wilson, 2000). Generally the production of the fruiting bodies starts after the death of the host plant tissue. Some of the endophytes produce bioactive compounds, which provide an advantage for the host. This has been reported for grasses belonging to the Poaceae. Only few endophytes have been extensively studied. However, in same cases unusual and valuable drugs are produced by these endophytic fungi (Strobel, 2002). The important anticancer drug taxol was first isolated from the plant *Taxus brevifolia* (Wani *et al.*, 1971). Later Stierle *et al.* (1995) isolated taxol from endophytic fungi (*Taxomyces andreanae*, *Pestalotiopsis microspora*) of the same plant. Rizzo *et al.* (1997) showed that the endophytic fungi of *Baccharis* species and not the plant are responsible for the production of roridins and verrucarins causing toxic effects in livestock.

For the Argentinean medicinal plant *Erythrina crista-galli* anti-inflammatory (Miño *et al.*, 2002) and antibacterial (Mitscher *et al.*, 1988) activities have been described. *E. crista-galli* can be found in the tropical and subtropical regions of America and it is commonly used as an ornamental plant. In Argentina the wood is used in infusions or decoctions as astringent, narcotic, and sedative (Toursarkissian, 1980). In the course of our studies on endophytes of medicinal plants we found that the majority of the fungi isolated so far from different collections of *E. crista-galli* belongs to the genus *Phomopsis.* This genus comprises more than 400 different species widely distributed as pathogens, endophytes or even symbionts of plants (Uecker, 1988). In our investigation of a possible contribution of fungal metabolites to the pharmacological activities of the plant a screening of *Phomopsis* isolates resulted in the identification of eight known and several new metabolites. Re-

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cently phomol, a polyketide lactone with interesting anti-inflammatory activities *in vivo* has been described (Weber *et al.*, 2004). Here we describe the taxonomy of 12 *Phomopsis* strains, their fermentation, and the isolation, biological activities and structure elucidation of several new metabolites.

## Materials and Methods

### *Producing organisms*

All *Phomopsis* strains were isolated from leaves and dead or living twigs of *Erythrina crista-galli.* The plant material was collected in Argentina (Table I). It was cut and surface-sterilized by immersion in 70% ethanol for 1 min, 5% NaOCl for 3 min and 70% ethanol for 30 s, followed by a wash in sterile distilled water. Samples were then cut into small fragments and plated onto 2% malt agar with penicillin G and streptomycin sulfate (each 200 mg/l). The mycelial cultures are deposited in the culture collection of the Institute of Biotechnology and Drug Research (IBWF), Kaiserslautern, Germany.

### *Taxonomy*

The different fungal isolates showed all characteristics of the genus *Phomopsis.* The species, however, could not be unequivocally determined. On YMG agar the strains form dark pycnidial conidiomata, in which both $\alpha$- and $\beta$-conidia are produced. The $\alpha$-conidia are hyaline, nonseptate, and elliptic while the $\beta$-conidia are hyaline, nonseptate, filiform, and curved. The methods for DNA extraction and ITS amplification have been described by Köpcke *et al.* (2002). The primers used for amplification were ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG) and ITS4 (5′-TCCTCCGCTTATTGATATGC) according to White *et al.* (1990). Their method was used with slight modifications: A GeneAmp PCR System 9700 was employed (Applied Biosystem, Foster City, CA, USA). The PCR amplification cycle consisted of 30 s at 94 °C, 1 min at 50 °C, and 1 min at 72 °C. PCR products were sequenced by MWG Biotech (Ebersberg, Germany) using the same primers as for the amplification. Each sequence was obtained in duplicate from each of two separate PCR amplifications. Database searches were performed with the FASTA function of the GCG Wisconsin Package.

### *Fermentation*

Fungi were grown in different media: YMG (4 g yeast extract, 10 g glucose, 10 g malt extract/l; pH 5.5); KGA (4 g dried mashed potatoes, 20 g glucose/l; pH 5.5); Czapek-Dox (2 g $NaNO_3$, 1 g $K_2HPO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4$, 30 g sucrose/l $H_2O_{deion.}$; pH 6.3); corn meal (10 g corn meal, 10 g glucose, 1.5 g $K_2HPO_4$, 0.5 g KCl, 0.5 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$/l; pH 5.8); double malt (40 g malt extract/l; pH 5.5).

For screening purposes fungi were grown at room temperature in 2 l Erlenmeyer flasks containing 1 l of medium on a rotary shaker (120 rpm). 5–10 pieces of mycelium from well-grown agar plates were used as inoculum. When the glucose was completely used up and the pH started to rise to values above 6.0, the culture fluid was separated from the mycelium by filtration. The culture broth was extracted with EtOAc, the organic phase dried with $Na_2SO_4$, concentrated *in vacuo* and the residue dissolved in MeOH.

Fermentations were carried out in a Biolafitte C6 fermentor containing 20 l of medium with aeration (3 l air/min) and stirring (120 rpm) at room temperature. A well-grown culture (250 ml) in a 500 ml Erlenmeyer flask (grown at room temperature and 120 rpm) in the same medium was used as inoculum. The culture fluid was separated from the mycelium by filtration. The culture broth was either extracted with EtOAc and the organic phase dried with $Na_2SO_4$ and concentrated *in vacuo* or passed through a HP21 column [elution of the metabolites with $H_2O$/MeOH (1:1), MeOH, then acetone].

### *Isolation of the compounds*

The crude extracts were applied onto a column containing silica gel (Merck 60, 0.063–0.2 mm) and eluted with cyclohexane, cyclohexane/ethylacetate (9:1, 3:1, 1:1 v/v), ethylacetate, ethylacetate/methanol (3:1, 1:1 v/v) and methanol. For analytical HPLC (HP 1090 Series U, Hewlett-Packard, Waldbronn) a RP-18 column (LiChrospher, 5 $\mu$m particle size, 125 × 4 mm, Merck, Darmstadt) was used (flow: 1.5 ml/min; gradient: $H_2O$/methanol 0–70% in 20 min, 70–100% in 10 min). For preparative HPLC a Jasco Model PU-1586 with a Multiwavelength-Detector MD-910 was used (column: Phenomenex, Luna RP 18, 10 $\mu$m; 250 × 21 mm; gradients: $H_2O$/methanol; flow: 10 ml/min).

### *Fermentation of Phomopsis sp. E01094 and isolation of phomopyronol* (**1**)

The strain E01094 was cultivated in 20 l of Czapek-Dox medium. After 11 d of fermentation the mycelium was separated from the culture broth and the culture fluid passed through a column (30 × 5.5 cm) containing Mitsubishi Diaion HP21 adsorber resin. Elution with $H_2O$/acetone (1:1) yielded 569 mg of crude extract. This was applied onto a column (2.5 × 10 cm) containing silica gel 60 (Merck). An enriched product (65 mg) was obtained after elution with 100% MeOH. Preparative HPLC (see above) yielded 27 mg of **1** (Fig. 1) eluting at 41% MeOH.

### *Fermentation of Phomopsis sp. E01105 and isolation of compounds **2** and **3***

Fermentations of *Phomopsis* sp. E01105 were carried out in 20 l fermentors containing Czapek-Dox medium. After 9 d the fermentation was stopped and the culture fluid was extracted by passing it through a column containing HP21. The crude product (125 mg) of the first fraction ($H_2O$/MeOH, 1:1) was separated by silica gel chromatography (see above). An enriched product (46 mg) was obtained after elution with 100% ethylacetate. Preparative HPLC (see above) yielded 9 mg of **3** (Fig. 1) eluting at 25% MeOH. From the second fraction of the HP21 column (100% MeOH; 478 mg), an intermediate product (73 mg) was obtained by silica gel chromatography (see above) and elution with cyclohexane/ethylacetate (1:1). Preparative HPLC (see above) yielded 25 mg of **2** (Fig. 1) eluting at 32% MeOH.

### *Fermentation of Phomopsis sp. E02011 and isolation of compounds **4**, **5**, **6**, **7** and **8***

*Phomopsis* sp. E02011 was grown in 20 l of YMG medium. After consumption of the carbon source, the fermentation was stopped and the culture filtrate was extracted with ethylacetate. After evaporation of the organic solvent 1.4 g of crude extract were obtained. This was applied onto a column (2.5 × 10 cm) containing silica gel 60 (Merck) and fractionated into four fractions by elution with cyclohexane/ethylacetate 3:1 (F1), cyclohexane/ethylacetate 1:1 (F2), ethylacetate (F3) and MeOH (F4). F1 (237 mg) was subjected to preparative HPLC (see above) yielding 143 mg of **4** (Fig. 1) eluting at 55% MeOH.

F2 (327 mg) was purified by preparative HPLC (see above) yielding 33 mg of **5** (Fig. 1) eluting at 31% MeOH, 60 mg of **6** eluting with 53% MeOH, 57 mg of **7** eluting with 61% MeOH, and 30 mg of **8** eluting with 65% MeOH.

### *Clavatol (**9**), 4-hydroxymellein (**10**), mellein (**11**), mevalonolactone (**12**), mevinic acid (**13**), nectriapyrone (**14**), phomol (**15**), scytalone (**16**), tyrosol (**17**) (Fig. 2)*

Known compounds were detected and identified by HPLC-DAD-MS (HP-LC/MSD-System Series 1100, Hewlett Packard) with a LiChroCART Supersphere 100 RP-18 column (125 × 2 mm; 4 $\mu$m particle size). A gradient $H_2O$/acetonitrile 0–100% in 20 min at a flow rate of 0.8 ml/min was applied. Retention times [min]: Clavatol, 10.9; 4-hydroxymellein, 6.67; mellein, 9.1; mevalonolactone, 2.9; mevinic acid, 14.0; nectriapyrone, 9.4; phomol, 13,3; scytalone, 6.7; tyrosol, 4.2.

### *Spectroscopic characterization of the isolated compounds*

$^1$H NMR (500 MHz) and $^{13}$C NMR (125 MHz) spectra were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probe head equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts ($\delta$) are given in ppm, and the coupling constants ($J$) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^1J_{CH}$ = 145 Hz and $^nJ_{CH}$ = 10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). Mass spectra were recorded with a LC-MS (HP 1100; APCI, positive/negative mode) and a Micromass Q-TOF MICRO instrument (HR electrospray spectra), while the UV and IR spectra were recorded with a Perkin-Elmer $\lambda$ 16 and a Bruker IFS 48 spectrometer. The optical rotation was measured with a Perkin-Elmer 141 polarimeter at 22 °C.

*Phomopyronol* (**1**): Colourless oil; $[\alpha]_D$ – 11° (MeOH, *c* 0.5). – UV (MeOH): $\lambda_{max}$ (log $\varepsilon$) = 296 nm (3.46). – IR (KBr): $\nu$ = 3410, 2930, 1680, 1565, 1465, 1250 and 1050 cm$^{-1}$. – $^1$H NMR

(500 MHz, $CDCl_3$): δ[mult., *J* (Hz)] = 6.06 (s, 5-H), 3.89 (s, 12-$H_3$), 3.66 (m, 9-Ha), 3.62 (m, 9-Hb), 2.84 (hext., *J* = 7, 7-H), 1.97 (m, 8-Ha), 1.91 (s, 10-$H_3$), 1.77 (m, 8-Hb), 1.28 (d, *J* = 7.2, 11-$H_3$). – $^{13}C$ NMR (125 MHz, $CDCl_3$): δ = 167.4 (C-6), 166.0 (C-2 and C-4), 101.2 (C-3), 93.6 (C-5), 60.2 (C-9), 56.2 (C-12), 37.2 (C-8), 35.4 (C-7), 18.4 (C-11), 8.4 (C-10). – HRESMS: 213.1102 ($C_{11}H_{17}O_4$ requires 213.1127).

*3-Phenylpropane-1,2-diol* (**2**): Colourless oil; $[\alpha]_D$ – 46° (MeOH, *c* 0.3). – UV (MeOH): $\lambda_{max}$ (log ε) = 260 nm (2.47). – IR (KBr): ν = 3390, 2925, 1605, 1495, 1455, 1090, 1030, 745 and 700 $cm^{-1}$. – $^1H$ NMR (500 MHz, $CDCl_3$): δ[mult., *J* (Hz)] = 7.32 (t, *J* = 7.5, 3-H and 5-H), 7.25 (t, *J* = 7.5, 4-H), 7.23 (d, *J* = 7.5, 2-H and 6-H), 3.96 (m, 8-H), 3.70 (dd, *J* = 3.2 and 11.2, 9-Ha), 3.54 (dd, *J* = 6.9 and 11.2, 9-Hb), 2.81 (dd, *J* = 5.4 and 13.7, 7-Ha), 2.77 (dd, *J* = 8.0 and 13.7, 7-Hb). – $^{13}C$ NMR (125 MHz, $CDCl_3$): δ = 137.7 (C-1), 129.3 (C-2/C-6), 128.7 (C-3/C-5), 126.6 (C-4), 73.0 (C-8), 66.0 (C-9), 39.8 (C-7). – HRESMS: 153.0929 ($C_9H_{13}O_2$ requires 153.0916).

*4-(2,3-Dihydroxypropoxy)benzoic acid* (**3**): Colourless oil; $[\alpha]_D$ – 23° (MeOH, *c* 0.6). – UV (MeOH): $\lambda_{max}$ (log ε) = 257 nm (4.27). – IR (KBr): ν = 3435, 2930, 1690, 1610, 1515, 1445, 1290, 1170, 1115, 1040, 960, 850 and 770 $cm^{-1}$. – $^1H$ NMR (500 MHz, $CDCl_3$): δ[mult., *J* (Hz)] = 7.86 (m, 3-H and 5-H), 6.78 (m, 2-H and 6-H), 4.26 (m, 8-$H_2$), 3.94 (m, 9-H), 3.65 (m, 10-Ha), 3.57 (dd, *J* = 5.9 and 11.6, 10-Hb). – $^{13}C$ NMR (125 MHz, $CDCl_3$): δ = 167.0 (C-1), 161.7 (C-7), 131.8 (C-3/C-5), 120.7 (C-4), 115.5 (C-2/C-6), 70.1 (C-9), 65.2 (C-8), 63.2 (C-10). – HRESMS: 213.0755 ($C_{10}H_{13}O_5$ requires 213.0763).

*2-(Hydroxymethyl)-3-propylphenol* (**4**): Colourless oil. – UV (MeOH): $\lambda_{max}$ (log ε) = 279 nm (3.81). – IR (KBr): ν = 3345, 2960, 1590, 1465, 1340, 1285, 1185, 990, 785 and 750 $cm^{-1}$. – $^1H$ NMR (500 MHz, $CDCl_3$): δ[mult., *J* (Hz)] = 7.11 (t, *J* = 8.0, 5-H), 6.74 (d, *J* = 8.0, 6-H), 6.72 (d, *J* = 8.0, 4-H), 4.92 (s, 7-$H_2$), 2.56 (t, *J* = 7.7, 8-$H_2$), 1.54 (m, 9-$H_2$), 0.96 (t, *J* = 7.4, 10-$H_3$). – $^{13}C$ NMR (125 MHz, $CDCl_3$): δ = 156.6 (C-1), 140.9 (C-3), 128.8 (C-5), 122.7 (C-2), 121.6 (C-4), 114.4 (C-6), 60.0 (C-7), 35.2 (C-8), 24.8 (C-9), 14.0 (C-10). – HRESMS: 167.1059 ($C_{10}H_{15}O_2$ requires 167.1072).

*2-(Hydroxymethyl)-3-(1-hydroxypropyl)phenol* (**5**): Colourless oil; $[\alpha]_D$ – 26° (MeOH, *c* 0.9). – UV (MeOH): $\lambda_{max}$ (log ε) = 280 nm (3.35). – IR (KBr): ν = 3380, 2965, 1590, 1465, 1270, 995, 875, 795 and 750 $cm^{-1}$. – $^1H$ NMR (500 MHz, $CDCl_3$): δ[mult., *J* (Hz)] = 7.16 (t, *J* = 7.9, 5-H), 6.97 (d, *J* = 7.9, 6-H), 6.78 (d *J* = 7.9, 4-H), 4.90 (d, *J* = 12.9, 7-Ha), 4.83 (d, *J* = 12.9, 7-Hb), 4.68 (t, *J* = 7.0, 8-H), 1.78 (m, 9-Ha), 1.74 (m, 9-Hb), 0.92 (t, *J* = 7.3, 10-$H_3$). – $^{13}C$ NMR (125 MHz, $CDCl_3$): δ = 156.7 (C-1), 142.5 (C-3), 129.1 (C-5), 123.2 (C-2), 118.2 (C-4), 115.9 (C-6), 73.2 (C-8), 58.3 (C-7), 30.8 (C-9), 10.5 (C-10). – HRESMS: 205.0837 ($C_{10}H_{14}O_3Na$ requires 205.0841).

*Compound* **6**: Colourless oil; $[\alpha]_D$ + 70° (MeOH, *c* 0.8). – UV (MeOH): $\lambda_{max}$ (log ε) = 216 nm (3.89). – IR (KBr): ν = 3440, 2935, 1705, 1675, 1460, 1250, 1200, 1165, 1080, 1005 and 870 $cm^{-1}$. – $^1H$ NMR (500 MHz, $CDCl_3$): δ[mult., *J* (Hz)] = 6.96 (dd, *J* = 5.7 and 9.9, 3-H), 6.04 (d, *J* = 9.9, 2-H), 5.11 (m, 13-H), 4.30 (m, 4-H), 2.82 (m, 5-H), 2.68 (m, 6-H and 7-$H_2$), 2.17 (m, 9-Ha), 2.09 (m, 12-Ha), 1.81 (m, 11-Ha), 1.64 (m, 10-Ha), 1.37 (m, 12-Hb), 1.31 (m, 10-Hb and 11-Hb), 1.27 (d, *J* = 6.7, 14-$H_3$), 1.19 (m, 9-Hb). – $^{13}C$ NMR (125 MHz, $CDCl_3$): δ = 199.8 (C-1), 171.6 (C-8), 145.9 (C-3), 130.7 (C-2), 73.4 (C-13), 67.0 (C-4), 45.8 (C-6), 38.6 (C-7), 36.8 (C-5), 30.2 (C-12), 22.8 (C-9), 22.7 (C-11), 22.2 (C-10), 18.4 (C-14). – HRESMS: 253.1401 ($C_{14}H_{21}O_4$ requires 253.1440).

*8-(Hydroxymethyl)-2,2-dimethyl-7-propylchroman-3-ol* (**7**): Colourless oil; $[\alpha]_D$ + 17° (MeOH, *c* 0.5). – UV (MeOH): $\lambda_{max}$ (log ε) = 283 nm (3.54). – IR (KBr): ν = 3400, 2960, 1580, 1380, 1260, 1065 and 995 $cm^{-1}$. – $^1H$ NMR (500 MHz, $CDCl_3$): δ[mult., *J* (Hz)] 6.91 (d, *J* = 7.7, 5-H), 6.69 (d, *J* = 7.7, 6-H), 4.81 (s, 9-H2), 3.52 (d, *J* = 10.0, 3-H), 2.77 (dd, *J* = 10.0 and 14.3, 4-Ha), 2.61 (d, *J* = 14.3, 4-Hb), 2.58 (m, 10-$H_2$), 1.55 (hext., *J* = 7, 11-$H_2$), 1.27 (s, 13-$H_3$), 1.24 (s, 14-$H_3$), 0.96 (t, *J* = 7.3, 12-$H_3$). – $^{13}C$ NMR (125 MHz, $CDCl_3$): δ = 154.8 (C-8a), 140.3 (C-7), 130.2 (C-5), 125.2 (C-8), 123.9 (C-4a), 121.6 (C-6), 80.6 (C-3), 73.1 (C-2), 59.0 (C-9), 35.2 (C-10), 33.7 (C-4), 26.2 (C-13), 24.9 (C-11), 23.0 (C-14), 14.0 (C-12). – HRESMS: 251.1642 ($C_{15}H_{23}O_3$ requires 251.1647).

*(4E,10E)-Trideca-4,10,12-triene-2,8-diol* (**8**): Colourless oil; $[\alpha]_D$ – 12° (MeOH, *c* 0.5). – UV (MeOH): $\lambda_{max}$ (log ε) = 225 nm (4.12). – IR (KBr): ν = 3410, 2925, 1650, 1450, 1075, 1005, 970 and 900 $cm^{-1}$. – $^1H$ NMR (500 MHz, $CDCl_3$): δ[mult., *J* (Hz)] = 6.33 (ddd, *J* = 10.1, 10.5 and 17.0, 12-H), 6.14 (dd, *J* = 10.5 and 15.2, 11-H), 5.70 (dt, *J* = 15.2 and 7.4, 10-H), 5.57 (dt, *J* = 15.7 and 6.1, 5-H), 5.48 (dt, *J* = 15.7 and 7.4, 4-H), 5.15 (d, *J* = 17.0, 13-Ha), 5.03 (d, *J* = 10.1, 13-Hb), 3.80

(hept., $J$ = 6.0, 2-H), 3.68 (m, 8-H), 2.32 (m, 9-Ha), 2.21 (m, 3-Ha), 2.20 (m, 9-Hb), 2.14 (m, 6-$H_2$), 2.08 (m, 3-Hb), 1.56 (m, 7-$H_2$), 1.19 (d, $J$ = 6.2, 1-$H_3$). – $^{13}$C NMR (125 MHz, $CDCl_3$): $\delta$ = 136.7 (C-12), 134.2 (C-11), 133.8 (C-5), 130.3 (C-10), 126.5 (C-4), 116.0 (C-13), 70.7 (C-8), 67.2 (C-2), 42.4 (C-3), 40.7 (C-9), 36.4 (C-7), 29.0 (C-6), 22.7 (C-1). – HRESMS: 233.1496 ($C_{13}H_{22}O_2Na$ requires 233.1517).

*Biological assays*

Antimicrobial activities were determined in the serial dilution assay as described previously (Anke *et al.*, 1989). Inhibition of growth of germinated seeds of *Setaria italica* and *Lepidium sativum* was tested according to Anke *et al.* (1989). Nematicidal activity was measured as described by Stadler *et al.* (1994).

Cytotoxic activity was assayed as described previously (Zapf *et al.*, 1995) with slight modifications. L1210 (ATCC CCI 219) and Colo-320 cells (DSMZ ACC144) were grown in RPMI 1640 medium (GIBCO, BRL), MDA-MB-231 (ATCC HTB26) cells in D-MEM (GIBCO, BRL), supplemented with 10% fetal calf serum (FCS) (GIBCO, BRL), 65 $\mu$g/ml of penicillin G and 100 $\mu$g/ml of streptomycin sulfate. The assays contained $1 \times 10^5$ cells/ml medium.

Reporter gene assays: The STAT1/STAT2 dependent signal transduction was tested in HeLa-S3 cells (ATCC CCL2.2). The reporter plasmid pGE3-GAS/ISRE contained five copies of a GAS/ISRE consensus oligonucleotide immediately upstream of the thymidine kinase promoter driven SEAP reporter gene (Erkel *et al.*, 1996). The TNF-$\alpha$ promotor-driven luciferase reporter plasmid pJR-TNF-pro was tested in Jurkat cells (ATCC TIB 152) as described by Weidler *et al.* (2000). The assay using the NF-$\kappa$B promotor-driven luciferase reporter plasmid pNF$\kappa$B-Luc (Stratagene) in Jurkat cells was performed as described for the TNF-$\alpha$ reporter gene assay with minor modifications: The cells were electroporated with 30 $\mu$g of the pNF$\kappa$B-Luc vector. After electroporation the cells were seeded in 24-well plates ($5 \times 10^7$ – $1 \times 10^8$ cells/ml in OPTIMEM medium containing 10% of fetal calf serum). The activity of the luciferase was determined 24 h after transfection using the Luciferase Assay System (Promega, Mannheim) according to the manufacturer's instructions with a luminometer.

Mouse ear edemas were induced with TPA according to Carlson *et al.* (1985) and De Young *et al.* (1989). The assay was performed as described before (Weber *et al.*, 2004). Male Swiss mice (25–30 g) were used in groups of 10 animals each. The right ear of each mouse received 2.5 $\mu$g of 12-O-tetradecanoylphorbol-13-acetate (TPA) by topical application as 0.125 $\mu$g/$\mu$l acetone solution (10 $\mu$l to each side of the ear). Compounds dissolved in acetone were applied topically immediately after TPA at a dose of 1 mg/ear. The left ear, used as a control, received only the vehicle. Indomethacin, an inhibitor of prostaglandin synthesis, was used as reference drug (0.5 mg/ear). After 4 h, the animals were killed and disks of 6 mm diameter were removed from each ear and weighted. The swelling was measured as the difference in weight between the punches from the right and left ears.

## Results and Discussion

*Isolation and identification of endophytic fungi*

The *Phomopsis* strains were isolated from fresh leaves and twigs as well as from dead plant material. *Phomopsis* spp. were isolated from all *Erythrina crista-galli* trees although the plant material was collected in different places and during different seasons (Table I). All twelve isolates develop black pycnidia on YMG agar. Within these pycnidia, $\alpha$- and $\beta$-conidia are produced. A comparison of the ribosomal RNA genes is commonly used for the phylogenetic analysis of related species (Köpcke *et al.*, 2002). Therefore the ITS sequences of seven *Phomopsis* strains were analyzed and compared to one of the strains (E01094) taken as a reference (Table II). The alignment of the ITS sequences showed that the sequences of E01094 and E02089 are identical. Since isolates with one to three base pair differences are considered to belong to the same species (Pfunder *et al.*, 2001) this is true for five strains, E01094, E02018, E02074, E02084, and E02089. Strain E02011 apparently belongs to another species. A FASTA search was performed for the strains E01094, E01105, and E02011. E01094 and E01105 have a sequence identity of 98.7% (8 differences) or 98.0% (10 differences) with *Phomopsis* sp. GJS83–377 (AF 102999). Strain E02011 has a sequence identity of 95.6% (26 differences) with *Diaporthe caulivola* 713. *Phomopsis* is the asexual stage of *Diaporthe*.

Table I. Endophytic fungi isolated from *Erythrina crista-galli*.

| Strain | Plant organ | Origin | Date |
|---|---|---|---|
| E01094 | Dead twig | Rio Capitan (tributary of the Parana) | Sept. 8, 2001 |
| E01105 | Green twig | Rio Capitan (tributary of the Parana) | Sept. 8, 2001 |
| E02001 | Leaves | Chivilcoy | Oct. 1, 2002 |
| E02003 | Green twig | Chivilcoy | Oct. 1, 2002 |
| E02004 | Dead twig | Chivilcoy | Oct. 1, 2002 |
| E02005 | Dead twig | Chivilcoy | Oct. 1, 2002 |
| E02011 | Dead twig | Parana-Delta | Oct. 1, 2002 |
| E02018 | Dead twig | Boraso-Delta | Oct. 1, 2002 |
| E02069 | Young twig | Campus of the University of Buenos Aires at the Rio de la Plata | Dec. 3, 2002 |
| E02074 | Young twig | Ruta Provincial 25 | Dec. 4, 2002 |
| E02084 | Young twig | Country road from Parana de las Palmas, 2 km from Port de Escobar | Dec. 4, 2002 |
| E02089 | Young twig | Rio Capitan (tributary of the Parana) | Dec. 5, 2002 |

Table II. Differences in the ITS sequences compared with the reference strain E01094.

| Strain | Differences in the ITS sequences |
|---|---|
| E01094 | Reference |
| E01105 | 4 substitutions |
| E02011 | 36 substitutions, 9 insertions, 1 deletion |
| E02018 | 1 deletion (T at position 543) |
| E02074 | 3 substitutions |
| E02084 | 2 substitutions |
| E02089 | – |

–, No differences.

### *Isolation and structure determination*

Phomopyronol (**1**)

Phomopyronol (**1**) (Fig. 1) was isolated from the culture fluid of the *Phomopsis* strain E01094 cultivated in 20 l Czapek-Dox medium, as described above. **1** is a new compound, and it is structurally related to the phomapyrones reported from the blackleg fungus (Pedras *et al.*, 1994). The structure elucidation of **1** was hampered by the fact that only 10 signals were observed in the $^{13}C$ NMR spectrum recorded in $CDCl_3$, although high resolution MS experiments revealed that the compound contains 11 carbon atoms. The elemental composition suggested by the mass spectra, $C_{11}H_{16}O_4$, gives the unsaturation index 4, and with three double bonds **1** should also contain one ring. COSY and HMBC correlations clearly revealed the saturated substructure, and HMBC correlations from 11-$H_3$ to C-6 and from 7-H to C-5 and C-6 showed that the saturated part is attached to

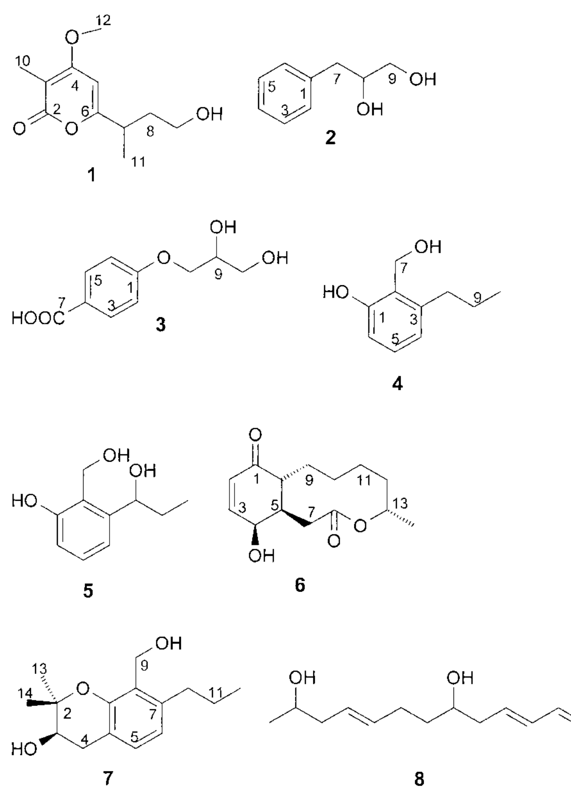

Fig. 1. Structures of phomopyronol (**1**), 3-phenylpropane-1,2-diol (**2**), 4-(2,3-dihydroxypropoxy)benzoic acid (**3**), 2-(hydroxymethyl)-3-propylphenol (**4**), 2-(hydroxymethyl)-3-(1-hydroxypropyl)phenol (**5**), compound **6**, 8-(hydroxymethyl)-2,2-dimethyl-7-propylchroman-3-ol (**7**), and (4*E*,10*E*)-trideca-4,10,12-triene-2,8-diol (**8**).

C-6 and indicated that C-5/C-6 is part of a double bond that is polarized by a heteroatom (oxygen) attached to C-6. The methoxy protons gave a strong HMBC correlation to C-4 and a weak to C-5, and 10-$H_3$ correlated to C-3 and C-4, suggesting that the C-5/C-6 double bond is conjugated with the C-3/C-4 double bond. With this at hand only one carbon and one oxygen atom remain, and in order to comply with the unsaturation index this must be a carbonyl group in a lactone. C-9 could not be involved in this lactone, the proton shifts of 9-$H_2$ were too high, and the only remaining possibility is the proposed pyrone structure. C-2 and C-4 consequently had identical chemicals shifts in $CDCl_3$, and their signal was actually stronger than expected. The overlap of C-2 and C-4 observed has previously been reported for similar 4-hydroxylated and 4-methoxylated pyrones (Pedras *et al.*, 1994).

### 3-Phenylpropane-1,2-diol (**2**) and 4-(2,3-dihydroxypropoxy)benzoic acid (**3**)

The two aromatic compounds **2** and **3** were isolated from *Phomopsis* E01105. The compounds were isolated as described above, and their structures determined by spectroscopic techniques. Both are new natural products. For **2**, the presence of a phenyl group was indicated by both $^1H$ and $^{13}C$ NMR, and COSY and HMBC correlations within the side chain as well as between the side chain and the phenyl group determined the structure. The MS data were in accord with the structure. Compound **3** has the elemental composition $C_{10}H_{12}O_5$ and has 5 unsaturations, and a *para* substituted benzene ring with an electron-releasing substituent was indicated by the NMR data. HMBC correlations from 8-$H_2$ to C-1 showed that this oxygen atom is present as an ether, and the glycerol moiety was easily established from NMR data. The remaining carboxylic group must be positioned as indicated in Fig. 1, and HMBC correlations from 3-H/5-H to C-7 confirmed this.

### 2-(Hydroxymethyl)-3-propylphenol (**4**), 2-(hydroxymethyl)-3-(1-hydroxypropyl)phenol (**5**), compound **6**, 8-(hydroxymethyl)-2,2-dimethyl-7-propylchroman-3-ol (**7**) and (4*E*,10*E*)-trideca-4,10,12-triene-2,8-diol (**8**)

The five compounds were isolated from the culture fluid of *Phomopsis* sp. E02011 as described above. While compounds **4**, **5**, **7** and **8** are new, compound **6** was recently reported from a *Penicillium* strain as a bacterial DNA primase inhibitor (Chu *et al.*, 2003). Although the NMR data reported for **6** in $CD_3OD$ are identical to those obtained by us, the NMR data in $CDCl_3$ recorded in this investigation are given in the experimental part for convenience. NMR data revealed that **4** is a 1,2,3-trisubstituted benzene, and HMBC correlations showed that the order of the substituents are hydroxyl, hydroxylmethyl and propyl. **5** is an oxidized version of **4**, and similar NMR experiments showed that the difference between the two is that the propyl group of **4** is a 1-hydroxylpropyl group in **5**. **7** has the composition $C_{15}H_{22}O_3$ (with 5 unsaturations) according to high resolution mass spectrometry experiments, it contains the same basic structure as **4** but is prenylated and close to a chroman structure. HMBC correlations from the two methyl singlets to C-2 and C-3 showed that the two oxidized carbon atoms are vicinal, and COSY as well as HMBC correlations from 3-H to 4-$H_2$ and C-4a revealed that the oxygen link between C-2 and C-8a closes the second ring. The triene **8** is a straight chain without rings, according to the composition suggested by the mass spectra, and both double bonds are *E*. The structure elucidation is straightforward and based on COSY correlations, although the recorded HMBC correlations are perfectly in accord with the suggested structure.

### Identification of known compounds from fermentations of the *E. crista-galli* endophytes

To compare the production of secondary metabolites, all isolates were cultivated in 1 l YMG medium in 2 l Erlenmeyer flasks. Known compounds were detected and identified with HPLC-DAD-MS (see Material and Methods). Table III gives an

Table III. Overview of metabolites so far detected in cultures of the endophytic fungi of *Erythrina crista-galli*.

| *Phomopsis* sp. | Compounds produced |
|---|---|
| E01094 | **1**, **9**, **10**, **11**, **14**, **15**, **16**, **17** |
| E01105 | **2**, **3**, **11**, **12**, **14**, **16**, **17** |
| E02001 | **11** |
| E02003 | **11** |
| E02004 | **11** |
| E02005 | **11** |
| E02011 | **4**, **5**, **6**, **7**, **8**, **11**, **13**, **14**, **17** |
| E02018 | **11**, **15** |
| E02069 | **11** |
| E02074 | **11** |
| E02084 | **11**, **17** |
| E02089 | **10**, **11**, **14** |

overview of all metabolites detected in the extracts of the culture broth of the twelve *Phomopsis* strains of *E. crista-galli.* Mellein (**11**) (Fig. 2) was detected in the crude extracts of the culture fluids of all isolates. It has been described from fermentations of *Aspergillus melleus* (Nishikawa, 1933), *Fusarium larvarum* (Grove and Pople, 1979), *Cercospora taiwanensis* (Carmarda *et al.*, 1976) and many other fungi. The compound has phytotoxic, antibacterial, and antifungal activities (Takeuchi *et al.*, 1992; Wenke, 1993). In the extracts of two strains 4-hydroxymellein (**10**) was detected. Nectriapyrone (**14**) and tyrosol (**17**), both common fungal metabolites, were identified in the crude extracts of four isolates. For nectriapyrone antibacterial activity against *Staphylococcus aureus* has been described (Nair und Carey, 1975). Tyrosol was isolated by Cross *et al.* (1963) as a fungal metabolite of *Gibberella fujikuroi* with phytotoxic activity (Devys *et al.*, 1976). Mellein and nectriapyrone were isolated by Claydon *et al.* (1985) from *Phomopsis oblonga*, commonly found on the bark of trees of the genus *Ulmus.* Trees infected by *P. oblonga* are protected from the attack of insects of the genus *Scolytus* (bark beetle) (Webber, 1981).

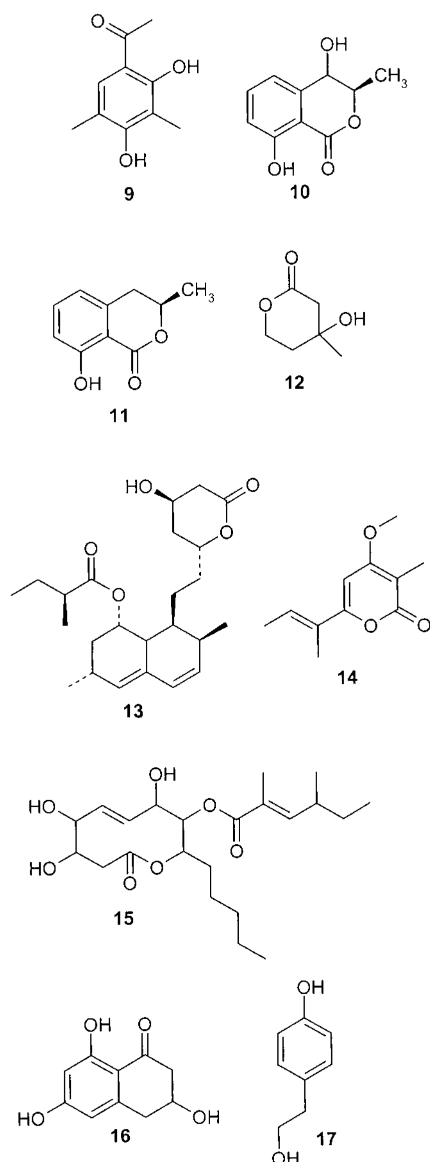


Fig. 2. Structures of the known compounds clavatol (**9**), 4-hydroxymellein (**10**), mellein (**11**), mevalonolactone (**12**), mevinic acid (**13**), nectriapyrone (**14**), phomol (**15**), scytalone (**16**), and tyrosol (**17**).

Scytalone (**16**) and phomol (**15**) (Weber *et al.*, 2004) were detected in two strains. Scytalone is an intermediate in the biosynthesis of melanin, the dark pigment of many phytopathogenic fungi (Bell and Wheeler, 1986).

Clavatol (**9**), mevalonolactone (**12**), and mevinic acid (**13**) were detected in only one endophyte. Clavatol was first isolated from cultures of *Aspergillus clavatus* (Hassal and Todd, 1947), but no biological activities have been described. Mevalonic acid is an intermediate in cholesterol biosynthesis. Mevinic acid is an inhibitor of HMG-CoA reductase, the key enzyme of cholesterol biosynthesis (Endo and Hasumi, 1997). Derivatives are used as cholesterol-lowering drugs.

*Biological properties*

The antimicrobial activities of the new compounds are shown in Table IV. None of the compounds inhibited the growth of *Bacillus brevis, B. subtilis*, *Micrococcus luteus*, *Mycobacterium phlei*, *Escherichia coli K12*, *Ascocyta pisi*, *Candida glabrata*, *C. krusei*, *C. parapsilosis*, *Fusarium fujikuroi* and *F. oxysporum* (MIC > 100 $\mu$g/ml).

Phytotoxic activities towards *Setaria italica* or *Lepidium sativum* were observed for **5**, **6**, **7**, and **8** at concentrations of 667 $\mu$g/ml (Table V). No nematicidal activities were observed at concentrations up to 100 $\mu$g/ml against *Meloidogyne incognita* and *Caenorhabditis elegans* (data not shown). Cytotoxic activities were tested up to a concentration of 100 $\mu$g/ml. Only compound **6**, showed significant (> 50 $\mu$g/ml) cytotoxic activities against

Table IV. Antimicrobial activities of compounds **1**–**8** in the serial dilution assay.

| Organism | Minimal inhibitory concentration [μg/ml] Compound | | | | | | |
|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **6** | **7** | **8** |
| Bacteria* | | | | | | | |
| *Arthrobacter citreus* | 100s | –* | – | – | 50s | – | – |
| *Corynebacterium insidiosum* | 50s | – | – | – | 50s | – | – |
| *Enterobacter dissolvens* | 50s | – | – | – | – | – | – |
| *Pseudomonas fluorescens* | 100z | – | – | – | 50s | – | – |
| Yeasts** | | | | | | | |
| *Nematospora coryli* | 100s | – | – | – | 50z | – | 50z |
| *Schizosaccharomyces octospora* | – | – | – | – | 100z | – | – |
| *Sporobolomyces roseus* | – | 100s | – | 100s | 20z | – | 100z |
| Fungi** | | | | | | | |
| *Absidia glauca* + | 50s | 50s | 10z | 50z | 10z | – | 50z |
| *Absidia glauca* – | 50s | 10 | 50s | 50z | 20z | 50z | 50z |
| *Aspergillus ochraceus* | – | – | – | 100z | 100z | – | – |
| *Paecilomyces variotii* | 10s | – | – | – | 50s | – | 50s |
| *Penicillium islandicum* | – | – | – | – | 100z | – | 50s |
| *Penicillium notatum* | 100s | – | – | – | 5s | – | – |
| *Zygorhynchus moelleri* | 100s | – | – | – | 100z | – | – |

* Nutrient broth (Difco).
** YMG-medium.
–, MIC > 100 μg/ml.
z, bactericidal/fungicidal.
s, bacteriostatic/fungistatic.

Table V. Phytotoxic activities against *Lepidium sativum* and *Setaria italica* at a concentration of 667 μg/ml.

| Compound | Inhibition of *Lepidium sativum* (%) | *Setaria italica* (%) |
|---|---|---|
| **1** | 50 | –* |
| **2** | – | – |
| **3** | – | – |
| **4** | 4 | 9 |
| **5** | 50 | 50 |
| **6** | 100 | 15 |
| **7** | 60 | 70 |
| **8** | 10 | 34 |

* No inhibition.

L1210 ($IC_{50}$ 1 μg/ml), Colo-320 ($IC_{50}$ 1 μg/ml), and MDA-MB-231 ($IC_{50}$ 5 μg/ml).

The transcription factors NFκB, STAT1/STAT2 and the cytokine TNF-α are key players in inflammation. The STAT1/STAT2 dependent signal transduction was tested in HeLa-S3 cells with a reporter plasmid containing five copies of a GAS/ISRE consensus oligonucleotide as described in Materials and Methods. The TNF-α promotor activity and NFκB dependent transcription were assayed in Jurkat cells as described above. At concentrations of 20 and 50 μg/ml no effects were observed for all compounds. Because of strong cytotoxic effects, **6** was not included in these assays.

In the mouse ear assay however, two compounds, mevinic acid and phomol, showed signifi-

Table VI. Topical anti-inflammatory activities in TPA-induced mouse ear edema.

| Substance | Edema [mg] (mean ± SEM) | Edema inhibition (%) |
|---|---|---|
| Control | 17.82 ± 0.71 | |
| Phomol (**15**) (1 mg/ear)* | 8.34 ± 1.11 | 53.20 |
| Mevinic acid (**13**) (1 mg/ear) | 8.64 ± 0.98 | 51.51 |
| Mellein (**11**) (1 mg/ear) | 13.85 ± 0.79 | 22.28 |
| Nectriapyrone (**14**) (1 mg/ear) | 14.3 ± 0.78 | 19.75 |
| Indomethacin (0.5 mg/ear) | 6.01 ± 0.69 | 66.00 |

* Weber *et al.*, 2004.

cant activities (Table VI). The anti-inflammatory effect of mevinic acids (statins) are clinically proven. Ongoing experiments will show if both compounds are present in the plant and contribute to its anti-inflammatory activities.

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