VOL. 57 NO. 9, SEPT. 2004 THE JOURNAL OF ANTIBIOTICS pp. 559 — 563

Phomol, a New Antiinflammatory Metabolite from an Endophyte of the Medicinal Plant *Erythrina crista-galli*

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(Received for publication June 15, 2004)

Phomol (**1**), a novel antibiotic, was isolated from fermentations of a *Phomopsis* species in the course of a screening of endophytic fungi from the medicinal plant *Erythrina crista-galli*. For this Argentinean leguminosa antiinflammatory and neuroleptic activities have been described. The compound exhibits antifungal, antibacterial and weak cytotoxic acticity. The antiinflammatory activity was tested in different reporter gene assays (TNF-α, STAT1/STAT2 and NF-κB) and in an ear edema model in mice. In the reporter gene assays **1** exhibited no activity, whereas **1** showed interesting antiinflammatory activity in the mouse ear assay. The compound is a polyketide lactone and its structure was elucidated by spectroscopic methods.

Erythrina crista-galli (Fabaceae) is widely distributed in tropical and subtropical regions of the American continent and is a popular ornamental plant in subtropical areas. In Argentina the wood of this medicinal plant is used in infusions or decoctions as astringent, narcotic, and sedative[1]. Antibacterial[2] and antiinflammatory[3] activities have been reported for this plant. Since *E. crista-galli* like all other plants harbours endophytic fungi, we became interested in a possible contribution of fungal metabolites to the pharmacological activities. The majority of endophytes isolated so far from different collections of *E. crista-galli* are species of the genus *Phomopsis*, which contains a large number of plant-inhabiting species[4]. A screening of these fungi resulted in the isolation of a new compound with antiinflammatory activities from fermentations of *Phomopsis* sp. E02018. Here we describe the taxonomy of the producing strain, the fermentation, isolation, biological activities and the structure elucidation of this metabolite which we have named phomol (**1**).

Materials and Methods

Producing Organism

Phomopsis sp. strain E02018 was isolated from a dead twig of *Erythrina crista-galli*. The plant material was collected at the Boraso Stream-Delta del Paraná, Argentina. A three-step ethanol, sodium hypochlorite, ethanol treatment was used to isolate the endophytic fungi. *Phomopsis* sp. strain E02018 showed all characteristics of the genus, the species, however could not be unequivocally determined. The strain exhibits dark pycnidial conidiomata, in which α- and β-conidia are produced. The α-conidia are hyaline, nonseptate, and elliptic while the predominant β-conidia are hyaline, nonseptate, filiform, and curved. Mycelial cultures are deposited in the culture collection of the LB Biotechnologie, Universitat Kaiserslautern.

Fermentation

Fermentations were carried out in 1 liter of KGA medium composed of: Dried mashed potatoes 0.4%,

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glucose 2%, pH 5.5. For solid media 1.5% agar was added. Fermentations were performed in Erlenmeyer flasks at room temperature on a rotary shaker (120 rpm). 5~10 pieces of mycelium from well-grown petri dishes were used as inoculum. During fermentation 50 ml samples were withdrawn and the culture broth separated 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 to a concentration of 10 mg/ml. The content of phomol (**1**) was determined by analytical HPLC of 25 μl samples (Merck Li Chrospher® 100 RP 18, 5 μm; column 125×4 mm; flow: 1.5 ml/minute; gradients: H_2O-methanol 0-70% in 20 minutes, 70-100% in 30 minutes; Rt phomol (**1**)=23.9 minutes).

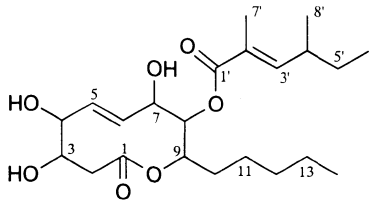
Isolation of the Compounds

After 39 days of fermentation the mycelia were separated from the culture broth. The culture fluid was extracted with an equal volume of ethylacetate. The organic phase was dried with Na_2SO_4 and concentrated *in vacuo*. The crude extract (282 mg) was applied onto a column (10×2.5 cm) containing silica gel (Merck 60, 0.063~0.2 mm). An enriched product (112.1 mg) was obtained after elution with 100% ethyl acetate. Preparative HPLC (Merck Lichrosorb® RP 18, 7 μm; column 250×25 mm; flow 7.5 ml/minute; gradients: H_2O-methanol 0-70% in 40 minutes, 70-100 % in 60 minutes) yielded 22 mg of phomol (**1**) (Rt=57.87 min).

Spectroscopy

^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probehead 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 (δ) 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 Jeol SX102 spectrometer, while the IR spectra were recorded with a Bruker IFS 48 spectrometer. The optical rotation was measured with a Perkin-Elmer 141 polarimeter at 22°C.

Fig. 1. Structure of phomol (**1**).



Phomol (**1**)

Phomol (**1**) was obtained as a colourless oil. $[\alpha]_D$ +37° (*c* 0.5 in $CHCl_3$). IR (KBr) 3420, 2960, 2930, 1710, 1270, 1230, 1150, 1100, and 750 cm^{-1}. ^1H NMR at 500 MHz in $CDCl_3$ (δ, mult., J in Hz) 6.60, dd, 1.2 and 10.0, 3′-H; 5.93, dd, 1.4 and 15.7, 6-H; 5.62, dd, 7.6 and 15.7, 5-H; 5.34, ddd, 2.8, 8.6 and 10.3, 9-H; 4.90, dd, 1.7 and 10.3, 8-H; 4.58, m 7-H; 3.96, m, 4-H; 3.95, m, 3-H; 2.62, d, 12.8, 2-Ha; 2.43, m, 4′-H; 2.29, dd, 11.0 and 12.8, 2-Hb; 1.86, s, 7′-H_3; 1.55, m, 10-Ha; 1.45, m, 10-Hb; 1.44, m, 5′-Ha; 1.36, m, 5′-Hb; 1.26, m, 11-H_2, 12-H_2 and 13-H_2; 1.01, d, 6.6, 8′-H_3; 0.87, t, 7.3, 6′-H_3; 0.85, t, 7.0, 14-H_3. ^{13}C NMR at 125 MHz in $CDCl_3$ (δ) 171.4 C-1, 167.0 C-1′, 150.0 C-3′, 132.6 C-6, 125.8 C-2′, 124.6 C-5, 79.4 C-4, 74.2 C-8, 72.6 C-3, 70.2 C-7, 68.1 C-9, 39.9 C-2, 35.0 C-4′, 31.4 C-13, 31.0 C-10, 29.5 C-5′, 23.8 C-11, 22.3 C-12, 19.5 C-8′, 13.9 C-14, 12.6 C-7′, 11.9 C-6′. HRFABMS $[M+H]^+$ *m/z* 413.2544 (required for $C_{22}H_{37}O_7$, 413.2539).

Biological Assays

Antimicrobial activity was determined in a serial dilution as described[5]. Inhibition of growth of germinated seeds of *Setaria italica* and *Lepidium sativum* was tested as described by ANKE *et al.*[5]

Cytotoxic activity was assayed as described previously[6] 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 μg/ml of penicillin G and 100 μg/ml of streptomycin sulfate. The assays contained 1×10^5 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 an GAS/ISRE consensus oligonucleotide immediately upstream of the thymidine kinase promoter driven SEAP reporter gene[7]. The TNF-α promotor-driven

luciferase reporter plasmid pJR-TNF-pro was tested in Jurkat cells (ATCC TIB 152) as described by WEIDLER *et al.*[8]. The assay using the NF-κB promotor-driven luciferase reporter plasmid pNFκB-Luc (Stratagene) in Jurkat cells was performed as described for the TNF-α reporter gene assay with slight modifications: The cells were electroporated with 30 μg of the pNFκB-Luc vector. After electroporation the cells were seeded in 24 well plates ($5\times10^7\sim1\times10^8$ cells/ml in OPTIMEM containing 10% of FCS). The activity of the luciferase was determined 24 hours 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.*[9] and DE YOUNG *et al.*[10]. Male Swiss mice (25~30 g) were used. The animals were housed in standard environmental conditions (25±1°C, with a 12 hours light/dark cycle) with free access to a standard commercial diet and water *ad libitum*. Groups of 10 animals each were used. The right ear of each mouse received 2.5 μg of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) by topical application as 0.125 μg/μl acetone solution (10 μl to each side of the ear). Compound **1** dissolved in acetone, was applied topically immediately after TPA at the dose of 1 mg/ear. The left ear, used as a control, received, only the vehicle. Indomethacin, an inhibitor of prostaglandins synthesis, was used as reference drug (0.5 mg/ear). After 4 hours, the animals were killed by cervical dislocation. Disks of 6 mm diameter were removed from each ear and the weight was determined. The swelling was measured as the difference in weight between the punches from the right and left ears. Results are expressed as mean±SEM. Differences between control and treated groups were tested for significance using a one-way analysis of variance (ANOVA) followed by Dunnett's test.

Results and Discussion

The fermentation of *Phomopsis* sp. E02018 was performed in KGA medium as described in the experimental section. The fermentation was terminated after 39 days when the free glucose was used up. The active compound were isolated by fractionation as described above, and characterized by NMR spectroscopy and mass spectrometry. In the LCMS spectra acquired with APCI, the ions *m/z* 413 was observed in the positive mode and 411 in the negative mode, indicating that the molecular weight of phomol (**1**) is 412. This was confirmed by HRFABMS, which in addition suggested that the composition of the compound is $C_{22}H_{36}O_7$. Examination of the 1D NMR spectra established this, as 22 carbon signals and signals integrating for 33 protons were observed. The structure was determined by 2D NMR experiments. A proton spin system extending from 2-H_2 all the way to 14-H_3 was indicated in the COSY spectrum, although the coupling constants were quite small in some instances. 7-H for example gives very small ^{1}H-^{1}H coupling constants to 6-H and 8-H, and to exclude the possibility that non-protonated groups are inserted between C-6 and C-7, or C-7 and C-8, the HMBC correlations in this moiety were investigated. Both 5-H and 6-H give HMBC correlations to C-7, 7-H gives HMBC correlations to C-5, C-6, C-8 and C-9, while 8-H gives HMBC correlations to C-7, C-9 and C-10. In addition, 8-H gives a HMBC correlation to C-1′, indicating that 8-OH is acylated, and this is supported by the chemical shift of 8-H (4.90 ppm). The nature of this acyl group was obvious from the HMBC correlations from 7′-H_3 to C-1′, C-2′ and C-3′, as well as COSY/HMBC correlations in the remaining part of the branched chain. The configurations of the C-2′/C-3′ double bond was shown to be *Z* by the NOESY correlation between 7′-H_3 and 4′-H and the lack of a NOESY coreelation between 7′-H_3 and 3′-H. 9-H gives, as expected, HMBC correlations to C-7, C-8, C-10 and C-11, but also to C-1. The chemical shifts for 9-H/C-9 (5.34/68.1 ppm) suggests that C-9 is acyloxylated, and as also 2-H_2 correlate to C-1 it is obvious that phomol (**1**) consists of a 10-membered lactone ring. Phomol (**1**) contains no less than six stereocentra, and although attempts to determine the relative configuration of the compound with NOESY experiments the configuration of C-4′ was not possible to decide. The structure of **1** is therefore given without any stereochemical details.

Biological Properties

Phomol (**1**) exhibited activity in the mouse ear model (Table 1), but at 50 μg/ml it did not show activity in any of the three reporter gene assays (data not shown). The compound exhibited antibacterial and antifungal activities (Table 2). It showed cytotoxic effects against all cell lines tested. Proliferation of the cells was reduced to 50% between 20 μg/ml (L1210) and 50 μg/ml (Colo-320, MDA-MB-231). No phytotoxic effects were observed against *Setaria italica* and *Lepidium sativum*. However, growth retardation was observed at concentrations starting from 330 μg/ml.

Table 1. Topical antiinflammatory activity of **1** in TPA induced mouse ear edema.

Substance	Edema [mg] mean ± SEM	Edema inhibition [%]
Control	17.82 ± 0.71	
1	8.34 ± 1.11	53.20
Indomethacin	6.01 ± 0.69	66.00

Table 2. Antimicrobial activity of **1** in the serial dilution assay.

Organism	MIC [μg/ml]	Incubation temperature [°C]
Bacteria (Difco Nutrient Broth)		
Arthrobacter citreus	20z*	27
Bacillus brevis	>100	37
Bacillus subtilis	>100	37
Corynebacterium insidiosum	10s**	27
Micrococcus luteus	>100	37
Mycobacterium phlei	>100	37
Escherichia coli K12	>100	37
Pseudomonas fluorescens	20z	37
Yeasts (YMG medium)		
Candida glabrata	>100	37
Candida krusei	>100	37
Candida parapsilosis	>100	37
Nematospora coryli	50z	27
Schizosaccharomyces octospora	20z	37
Sporobolomyces roseus	100s	27
Fungi (YMG medium)		
Absidia glauca +	5z	27
Absidia glauca -	10z	27
Ascocyta pisi	20s	27
Aspergillus ochraceus	50z	27
Fusarium fujikuroi	100z	27
Fusarium oxysporum	50s	27
Paecilomyces variotii	5s	37
Penicillium islandicum	10z	27
Penicillium notatum	20s	27
Zygorhynchus moelleri	10z	27

* z: bactericidal/fungicidal; ** s: bacteriostatic/fungistatic

Acknowledgments

This work was supported by the Volkswagen-Stiftung, the Fond der Chemischen Industrie, the Bundesministerium für Bildung und Forschung, and SETCIP.

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