Naphthoquinone spiroketal with allelochemical activity from the newly discovered endophytic fungus *Edenia gomezpompae*

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Abstract

Chemical investigation of the mycelium of *Edenia gomezpompae*, a newly discovered endophytic fungus isolated from the leaves of *Callicarpa acuminata* (Verbenaceae) collected from the ecological reserve El Eden, Quintana Roo, Mexico, resulted in the isolation of four naphthoquinone spiroketals, including three new compounds and palmarumycin CP2 (4). We elucidated the structures of the metabolites by extensive NMR spectroscopy studies, including DEPT, COSY, NOESY, HSQC, HMBC, and chiroptical methods. The trivial names proposed for these compounds are preussomerin EG1 (1), preussomerin EG2 (2) and preussomerin EG3 (3). In addition, the X-ray data for 4 were obtained. The bioactivity of the mycelial organic extracts and the pure compounds was tested against three endophytic fungi (*Colletotrichum* sp., *Phomopsis* sp., and *Guignardia manguifera*) isolated from the same plant species (*C. acuminata*, Verbenaceae) and against four economically important phytopathogenic microorganisms (two fungoid oomycetes, *Phythophthora capsici* and *Phythophthora parasitica*, and the fungi *Fusarium oxysporum* and *Alternaria solani*). Spiroketals 1–3 displayed significant growth inhibition against all the phytopathogens. IC50 values for the four phytopathogens were from 20 to 170 μg/ml. Palmarumycin CP2 (4) was not bioactive against any of the fungi tested. Compound 1 showed the strongest bioactivity. The acetylated derivatives of preussomerin EG1 (1), 1a and 1b, were obtained and their biological activity was tested on endophytes and phytopathogens. Preussomerin EG1, 1a and 1b exhibited significant bioactivity against all microorganisms tested with the exception of *Alternaria solani*. This is the first report of allelochemicals with antifungal activity from the newly discovered endophytic fungus *E. gomezpompae*.

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Keywords: *Edenia gomezpompae*; Endophytic fungi; *Callicarpa acuminata*; Verbenaceae; Allelochemicals; Naphthoquinone spiroketal; Preussomerin EG1; Preussomerin EG2; Preussomerin EG3; Palmarumycin CP2; Phytopathogenic fungi; Phytopathogenic fungoid oomycetes

1. Introduction

Endophytes are microbes that colonize living internal tissues of plants without causing any immediate, overt, negative effects or external symptoms. They are presumably ubiquitous in the plant kingdom. Colonization and
propagation of endophytes may offer a significant benefit to their host plants by producing a plethora of metabolites that provide protection and survival value to the plants. These compounds could impact the broader ecological community as plant growth regulators, antimicrobials, antivirals, and insecticidals, or even mediate resistance to some types of abiotic stress. Endophytes also could be potential sources of novel natural products with agrochemical, pharmaceutical, and industrial potential (Strobel, 1996, 2006; Rodriguez, 1996; Strobel and Long, 1998; Bacon and White, 2000; Tan and Zou, 2001; Schulz et al., 2002; Strobel and Daisy, 2003; Strobel et al., 2004; Stone et al., 2004; Wiyakrutta et al., 2004; Gunatilaka, 2006).

Among the large number of novel bioactive metabolites that are known from various fungi, the preussomerins and palmarumycins (deoxypreussomerins) belong to a relatively new and rare family of bioactive natural products based on a 1,8-dihydroxynaphthalene derived spiroketal unit linked to a second, oxidized naphthalene moiety (Weber and Gloer, 1991; Krohn et al., 2001; Hu et al., 2006; Jiao et al., 2006). These compounds exhibit an elaborate range of hydroxylation, oxidation, and unsaturation patterns. Preussomerins and deoxypreussomerins possess a wide range of biological properties, including antibacterial, antifungal, algicidal, herbicidal, antiplasmodial, and anti-tumor activities (Weber et al., 1990; Weber and Gloer, 1991; Krohn et al., 1994a,b, 2001; Soman et al., 1999; McDonald et al., 1999; Bode et al., 2000; Lazo et al., 2001; Wipf et al., 2001a; Seephonkai et al., 2002; Krohn, 2003; Prajoubklang et al., 2005). Some of these compounds have been identified as novel inhibitors of ras-farnesyltransferase (Singh et al., 1994; Vilella et al., 2000), DNA gyrase, topoisomerase II (Sakemi et al., 1995) and thioreredoxin–thioredoxin reductase (Wipf et al., 2001b, 2004, 2005) and thus are of interest in terms of their potential in cancer chemotherapy.

As a part of a screening program directed towards the isolation of biologically active metabolites from plant species and endophytic fungi from the Ecological Reserve El Eden, Quintana Roo, Mexico (Anaya et al., 2003a,b, 2005; Macías-Rubalcava et al., 2007), we have investigated the allelochemical potential from the newly discovered endophytic fungus Edenia gomezpompae. This fungus belongs to a new species within the family Pleosporaceae (Order Pleosporales). Morphological, physiological, and molecular studies indicated that it belongs to a new genus and species (González et al., 2007).

In the present paper, we report the isolation and structure elucidation of the major bioactive compounds from Edenia gomezpompae: the new naphthoquinone spiroketal preussomerin EG1 (1), preussomerin EG2 (2), and preussomerin in EG3 (3), and the known palmarumycin CP2 or deoxypreussomerin B (4). In addition, we also present the results of antagonism tests of E. gomezpompae vs. phytopathogenic microorganisms and endophytic fungi, and the bioactivity of the four isolated and identified compounds against these microorganisms.

2. Results and discussion

The results presented come from the confrontation experiments to test for antagonisms. E. gomezpompae clearly inhibited the growth of the three other endophytic fungi: Colletotrichum sp., Phomopsis sp. and Guignardia mangiferae that were isolated from Callicarpa acuminata. In the presence of G. mangiferae, Edenia shows a tendency to change its morphology, and G. mangiferae produces a darker color (Fig. 1). Four economically important phytopathogens: Phytophtora parasitica and Phytophtora capsici (Oomycota), and Fusarium oxysporum and Alternaria solani (Eumycota), also were inhibited by the presence of E. gomezpompae, particularly the two species of Phytophtora (Fig. 2).

E. gomezpompae was cultivated using fermentation in potato dextrose liquid medium (PD). Organic extracts of both the culture medium and mycelium showed significant activity against the endophytic fungi Phomopsis sp. and Colletotrichum sp. (Fig. 3a), but the culture medium extract had no effect against G. mangiferae growth; however, this fungus was significantly inhibited by the mycelial extract of E. gomezpompae. The culture medium extract significantly inhibited the radial growth of the four phytopathogenic species (Fig. 3b). P. capsici and P. parasitica were inhibited 100% by the culture medium extract. The mycelial extract also significantly inhibited the growth of these two species but did not significantly inhibit A. solani or F. oxysporum.
The culture medium extract was more active against *P. capsici* and *P. parasitica* (IC$_{50}$ = 47.8 and 125.9 µg/ml, respectively), while the mycelial extract was more active against *P. parasitica* and *Phomopsis* (IC$_{50}$ = 169.8 and 313.5 µg/ml, respectively). The IC$_{50}$ value of culture medium for *P. capsici* was similar to the IC$_{50}$ value of the fungicide Captan® (Table 1). Considering these results and the extract yields obtained from the mycelium and culture medium (14.25 g and 830 mg, respectively), we selected the mycelial extract for the bioassay-guided fractionation.

Bioassay-guided fractionation of the mycelial extract from *E. gomezpompae* led to the isolation of four naphthoquinone spiroketal derivatives. These compounds included three new preussomerins that were characterized by...
isolated from phytopathogens with economic importance and known endophytic fungi. Callicarpa acuminata N

Table 1
Bioactivity of the culture medium and mycelial extracts from endophytic fungus Edenia gomezpompae on diameter growth (IC50, μg/mL) of phytopathogens with economic importance and known endophytic fungi isolated from Callicarpa acuminata.

<table>
<thead>
<tr>
<th>Test organisms</th>
<th>Culture medium extract</th>
<th>Mycelial extract</th>
<th>Captan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytopathogens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alternaria solani</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>22.9</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>380.2</td>
<td>&gt;500</td>
<td>28.4</td>
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<tr>
<td>Phomopsis capsici</td>
<td>47.8</td>
<td>458.3</td>
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</tr>
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<td>Phomopsis parasitica</td>
<td>125.9</td>
<td>169.8</td>
<td>12.1</td>
</tr>
<tr>
<td>Endophytic fungus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colletotrichum sp.</td>
<td>373.8</td>
<td>&gt;500</td>
<td>40.1</td>
</tr>
<tr>
<td>Phomopsis sp.</td>
<td>216.3</td>
<td>313.5</td>
<td>4.7</td>
</tr>
<tr>
<td>Guignardia mangifera</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>51.9</td>
</tr>
</tbody>
</table>

IC50 (the effective dose for 50% diameter growth reduction).

* Results after 4 days of incubation.
* Results after 3 days of incubation.
* Results after 10 days of incubation.

Analysis of preussomerin EG1 (1) by HREIMS and 13C NMR spectroscopy indicated that it has the molecular formula C20H12O6 (15 degrees of unsaturation). The infrared spectrum of 1 showed absorption bands for a conjugated carbonyl at 1642 cm⁻¹, an αβ-unsaturated carbonyl group at 1679 cm⁻¹ and an hydroxyl group at 3078 cm⁻¹. The 1H NMR spectrum (Table 2) contained resonances indicating the presence of 1,2,3-trisubstituted and 1,2,3,4-tetrasubstituted aromatic rings. These resonances correspond to two sets of o-related aromatic protons at δH 6.61 (d J = 9.0 Hz, H-8) and 7.01 (d J = 9.0 Hz, H-7) ppm and δH 6.56 (d J = 9.5 Hz, H-2') and 7.71 (d J = 9.5 Hz, H-3') ppm. These last proton resonances (δH 6.56 and 7.71) are typical of a Z-olefin fragment that could belong to an αβ-unsaturated carbonyl group. In addition, these spectra exhibited the presence of a hydrogen atom of a hydroxyl group at δH 11.71 (OH-9) ppm, which is chelated with a carbonyl group and slowly exchanges with deuterium upon shaking with D2O. Two methylene groups between δH 2.53 and 3.35 (H a,b-2 and H a,b-3) ppm, and three aromatic protons between δH 7.03-7.59 (H-7, H-8' and H-9') ppm, were assigned based on the connections observed in the 1H-1H COSY and 1H-13C NOESY experiment (Table 3). The 13C NMR spectrum (Table 2) of 1 exhibited 20 carbon signals which were assigned with the help of an HSQC experiment. Two of the quaternary carbon signals appeared at δC 89.6 (C-4') and 93.7 (C-4) ppm, that are characteristic of the spiroketals carbons of the preussomerins (Weber et al., 1990; Weber and Gloer, 1991; Singh et al., 1994; Krohn et al., 2001; Quesada et al., 2004). The other carbons were identified as two conjugated carbonyls appearing at δC 184.07 (C-1') and 201.9 (C-1) ppm and seven olefinic or aromatic methines, one of which appeared at δC 141.2 (C-3') ppm and was assigned to a carbon β to the carbonyl group. Two carbon signals at δC 33.7 and 32.6 ppm correspond to the methylene groups C-2 and C-3, respectively. The remaining seven carbons are quaternary and were assigned as shown in Table 2. The structure of 1 was also supported by the HMBC experiment of the molecule (Fig. 4).

HREIMS and 13C NMR analysis of preussomerin EG2 (2) gave a molecular formula of C20H14O7 (14 degrees of unsaturation). The 1H NMR spectrum (Table 2) again contained resonances for the characteristic trisubstituted and tetrasubstituted aromatic rings. Comparison of the 1H NMR spectrum of 2 with that of preussomerin EG1 (1) indicated absence of the C-2', C-3' double bond, the presence of a hydroxyl group at H-3'/C-3' (δH/δC 2.38/70.3 ppm), and an apparent triplet of an oxygenated methine proton at δH 4.71 (1H, pseudo-t dd, J = 3.0, 3.0 Hz, H-3') ppm as well as two self-coupled aliphatic proton signals at δH 3.02 (J = 3.0, 18.3 Hz; H-2'a) and 3.38 (J = 3.0, 18.3 Hz, H-2'b) ppm, which in turn were coupled...
to the methine proton and correlated with a hydroxyl group in NOESY spectra (Table 3).

The α configuration was assigned to the stereogenic center at C-3 based on the following observation. The small coupling constant of 3.0 and 3.0 Hz between H-3′ and two methylene protons H-2a′ and H-2b′ in the 1H NMR spectrum of preussomerin E2 suggests that H-3′ must be placed in the pseudo-equatorial position and thus the hydroxyl group must be placed in a pseudo-axial position of the pseudochair ring conformation. (Weber and Gloer, 1991; Singh et al., 1994; Soman et al., 1999; Seepkonkai et al., 2002). This observation was strongly supported by application of chiroptical methods for the assignment of the absolute configuration. This method was previously applied successfully to elucidate the absolute configurations of several palmarumycins and preussomerins. The preussomerins are ideal substrates for this kind of determination since they are conformationally very rigid molecules, fixed by their bisspiro structure (Bringmann et al., 1997; Krohn et al., 1997a,b, 2001). The CD spectrum of preussomerin EG2 (2) showed a negative n → π* Cotton effect around 334 and 218 nm, supporting the depicted 3′R absolute configuration (Krohn et al., 2001).

Preussomerin EG3 (3) gave a molecular formula C_{23}H_{16}O_{7} (14 degrees of unsaturation) as deduced from HREIMS in agreement with the number of resonances for carbon and hydrogen atoms, detected in the NMR spectra. In fact, the 1H NMR and 13C NMR spectrum of preussomerin EG3 (3) are almost identical to that of preussomerin EG2 (2), except for the absence of free hydroxyl group at C-3′ and the presence of a methoxy group at δH 3.39 ppm. Analysis of HMBC data, chemical shifts, and 1H NMR J values verified the structure of 3 as a 3′-O-methyl analogue of preussomerin EG2 (2). The data of the 1H and 13C NMR spectra, together with the correlations resulting from the 1H–1H NOESY and HMBC experiments, are listed in Tables 2 and 3. Once more, the absolute stereochemistry at C-3′ was assigned based on the negative Cotton effect at 334 nm in the CD spectrum. Consequently, the configuration at C-3′ was established as R (Krohn et al., 2001).

The new preussomerins 1, 2, and 3 differ from other known natural preussomerins by the missing epoxide in position 2 and 3 (Singh et al., 1994; Soman et al., 1999; Seepkonkai et al., 2002). The spectral properties of the known palmarumycin CP2 (4), including IR, 1H NMR, and 13C NMR spectroscopic data, were identical to those previously described in the literature (Krohn et al., 1994a,b; Singh et al., 1994; Ragot et al., 1999; Barrett et al., 2002). X-ray data of palmarumycin CP2 (4) were not published previously; therefore, we are describing X-ray data of this compound in the present study. The structure and

### Table 2

<table>
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<tr>
<th>Position</th>
<th>Preussomerin EG1 (1)</th>
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<th>Preussomerin EG3 (3)</th>
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<td>1</td>
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<td>3.37 (1H, ddd 18.6, 13.5, 5.5)</td>
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<td>(1^1)H and (1^3)C NMR spectroscopic data for preussomerins EG1, EG2 and EG3 isolated from (Edenia gomezpompa)(^{a,b})</td>
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\(^{a}\) 1H and \(1^3\)C NMR spectra were acquired in CDCl\(_3\) at 500 and 125 MHz, respectively; TMS was used as internal standard; chemical shifts are shown in the \(\delta\) scale with \(J\) values (Hz) in parentheses.

\(^{b}\) Assignments are based on DEPT, HMBC, HSQC, 1H–1H COSY and 1H–1H NOESY experiments, and chemical shift values.
Table 3
HMBC and NOESY spectroscopic data for Preussomerins EG₁, EG₂ and EG₃ isolated from Edenia gomezpompea

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</table>

* HMBC and NOESY spectra were acquired in CDCl₃ at 500 MHz; TMS was used as internal standard; chemical shifts are shown in the δ scale.

Fig. 4. Key ¹³C–¹H long range correlations observed in HMBC spectra for preussomerin EG₁ (1).

Fig. 5. An ORTEP drawing is displayed.

The natural compounds 1–4 and their derivatives (1a and 1b) were tested for their bioactivity against the two phytopathogenic fungi (F. oxysporum and A. solani), and the phytopathogenic fungoid oomycetes P. parasitica and P. capsici. Compound 1 and its derivatives were tested on the three endophytic fungi (Colletotrichum sp., Phomopsis sp., and G. mangifera). The results of these bioassays are shown in Figs. 6 and 7. Preussomerins 1–3 exhibited significant inhibitory activity against all phytopathogenic fungi at 100 μg/ml. In contrast, palmarubicin CP₂ (4) did not show significant antifungal activity. It is possible to see a continuum between the potency for growth inhibition for compounds 1–4 (Fig. 6). Preussomerin EG₁ (1) exhibited the strongest bioactivity causing complete growth inhibition of P. parasitica, P. capsici and F. oxysporum. A. solani was not inhibited by 1; however, compounds 2 and 3 inhibited its growth significantly (Fig. 6). The IC₅₀ values of the isolated compounds on the radial growth of phytopathogenic test microorganisms show that compounds 1–3 reduced the diameter growth of all target species in a concentration-dependent manner. Preussomerin EG₁ (1) was shown to be more active than the commercial fungicide Captan in the growth of P. capsici (Table 4).
Fig. 5. ORTEP drawing of palmarumycin CP 2 (4).

Fig. 6. Diameter growth-inhibitory activity of the isolated compounds (100 μg/ml) from endophytic fungus Edenia gomezpompae against phytopathogens A. solani, F. oxysporum, P. parasitica and P. capsici. Vertical bars represent SD, N = 4; *P < 0.05.

Table 4
Bioactivity of the isolated compounds from endophytic fungus Edenia gomezpompae on the diameter growth (IC50, M) of Alternaria solani, Fusarium oxysporum, Phytophthora parasitica and Phytophthora capsici

<table>
<thead>
<tr>
<th>Compound</th>
<th>A. solani</th>
<th>F. oxysporum</th>
<th>P. parasitica</th>
<th>P. capsici</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;5.75 × 10^{-4} M</td>
<td>1.45 × 10^{-4} M</td>
<td>57.71 × 10^{-5} M</td>
<td>5.61 × 10^{-5} M</td>
</tr>
<tr>
<td>2</td>
<td>&gt;5.46 × 10^{-4} M</td>
<td>3.11 × 10^{-4} M</td>
<td>1.58 × 10^{-5} M</td>
<td>2.14 × 10^{-5} M</td>
</tr>
<tr>
<td>3</td>
<td>&gt;5.26 × 10^{-4} M</td>
<td>4.47 × 10^{-5} M</td>
<td>1.95 × 10^{-5} M</td>
<td>&gt;5.26 × 10^{-5} M</td>
</tr>
<tr>
<td>4</td>
<td>&gt;6.29 × 10^{-4} M</td>
<td>&gt;6.29 × 10^{-5} M</td>
<td>&gt;6.29 × 10^{-5} M</td>
<td>&gt;6.29 × 10^{-5} M</td>
</tr>
<tr>
<td>Captan</td>
<td>7.62 × 10^{-5} M</td>
<td>4.46 × 10^{-5} M</td>
<td>4.04 × 10^{-5} M</td>
<td>1.53 × 10^{-4} M</td>
</tr>
</tbody>
</table>
Considering that preussomerin EG1 (1) was the most abundant natural product isolated from the mycelial extract (3.4 mg/l of culture medium) and it displayed the highest antifungal activity on almost all the fungi tested, we semi-synthesized two acetylated derivatives of preussomerin EG1 (1). Treatment of preussomerin EG1 (1) with Ac2O/pyridine afforded a monoacetylpreussomerin (1a) and a diacetylated product (1b). Diacetylpreussomerin 1b was obtained by addition of acetate in a Michael addition to the enone system of 1. Monoacetylpreussomerin (1a) significantly inhibited the four phytopathogens (1). Diacetylpreussomerin EG1 (1b) significantly inhibited F. oxysporum, P. capsici, and P. parasitica but did not inhibit A. solani. Results of bioassays of compound 1 and its two derivatives against endophytic fungi (Fig. 7) showed that the three compounds (100 µg/ml) significantly inhibited the mycelial growth of the three endophytic fungi, however Colletotrichum sp. was the most inhibited (100%).

3. Concluding remarks

Considering the results, some structure–activity relationships within this class of antifungal agents were established. When comparing the chemical structure of the preussomerin EG1 (1) with 2 and 3 and the acetylated derivatives 1a and 1b, the presence of the C-2′, C-3′ double bond is evident in the most bioactive compounds (1 and 1a). The absence of this double bond in compounds 2, 3, and 1b possibly determined a less inhibitory effect. This observation indicates that the presence of a hydroxyl (2), methoxy (3), and acetyl groups (1b) at C-3′ possibly diminished the bioactivity. On the other hand, a positive effect of a hydroxyl group at C-9 was evident by comparing the activities of 1 and 1a. Both compounds differ by the presence of the OH-9 or OCOCH3-9, respectively. In conclusion, the structure–activity relationship reveals that the hydroxyl group at C-9 and the presence of the C-2′, C-3′ double bond are possibly responsible for the higher bioactivity of 1.

Little biology is known regarding endophytes in tropical forest trees, where their abundance and diversity are thought to be greatest. Arnold et al. (2001) explored the occurrence of endophytes in a broad diversity of woody, angiospermous taxa in a lowland, moist tropical forest in central Panama. Fungal endophytes were found in every plant species examined and appear to be important, but largely immeasurable components of fungal biodiversity. Schulz et al. (1999) and Schulz and Boyle (2005) mentioned that there are no neutral interactions, but rather that endophyte-host interactions involve a balance of antagonisms, irrespective of the plant organ infected. There is always at least a degree of virulence on the part of the fungus, which enables infection of the host, whereas defense by the plant host limits colonization by fungal invaders and development of disease. It is also hypothesized that the endophytes, in contrast to known phytopathogens, generally have far greater phenotypic plasticity with regard to infection, local but also extensive colonization, latency, virulence, pathogenicity and (or) saprophytism. This phenotypic plasticity is suggested to be a motor of evolution.

The present study contributes to the knowledge of endophytic fungi of C. acuminata, a Verbenaceous shrub species from the low and medium tropical forest of Yucatan peninsula. E. gomezpompae has never been chemically explored, and this is the first report on bioactive compounds from this fungus. Results on bioactivity of the spiraketal type compounds that it produces lead us to hypothesize that they could be involved in the antagonistic balance between plant and fungi defenses.

Further studies on endophytic fungi in plant communities of this zone will contribute valuable information on the endophytic fungal species associated with rich plant diversity in natural conditions. New species of fungi are likely to be discovered, and bioprospecting studies on these fungi may allow us to discover new natural products or allelochemicals with agrochemical and pharmacological potential.

4. Experimental

4.1. General experimental procedures

Melting points were measured in a Fisher–Johns apparatus and are uncorrected. The IR spectra were obtained using KBr disks on a Perkin–Elmer 599-B spectrophotometer. UV spectra were obtained on a Shimadzu 160 UV spectrometer in MeOH solution. CD spectra were performed on a JASCO 720 spectropolarimeter at 25 °C in MeOH solution. Optical rotations were taken on a JASCO DIP 360 polarimeter. NMR spectra including COSY spectra, NOESY, HMBC and HMQC experiments were recorded on a Bruker DMX500, in CDCl3, either at 500
incubation; P. capsici
dish was sealed with Parafilm
test fungus then was added to the other side of the dish. The
performed on pre-coated silica gel 60 F 254 plates (Merck).
230 mesh, Merck). Analytical and preparative TLC were
fractionation. Open column chromatography (CC): silica gel 60 (70–
fractometer equipped with graphite-monochromated radia-
tion. Extensive TLC (CH$_2$Cl$_2$–MeOH; 99.05:0.5) of fraction F-
mycin EG 3 (7.4 mg). Bioactive fraction F-IX, eluted
with CH$_2$Cl$_2$–MeOH (97:3) was applied to a Si gel (60 g)
9:1). Eight
\[ n \times \text{hexane–CH}_2\text{Cl}_2 \] (3:7), spontaneously crystallized preussomerin
EG$_1$ (1) (24.1 mg). Primary fraction VII (eluted with
CH$_2$Cl$_2$), was resolved by successive preparative TLC
(CH$_2$Cl$_2$ × 3) to yield preussomerin EG$_1$ (1) (43.9 mg).
Extensive TLC (CH$_2$Cl$_2$–MeOH; 99:05:0.5) of fraction F-
VIII eluted with CH$_2$Cl$_2$–MeOH (99:1) yielded palmarumycin EG$_3$ (3) (26.4 mg) (Krohn et al.,
1994a,b; Singh et al., 1994; Ragot et al., 1999; Barrett et
al., 2002). From primary fraction V (eluted with
\[ n \times \text{hexane–CH}_2\text{Cl}_2 \] (3:7), bioactive fraction F-I, eluted
with CH$_2$Cl$_2$–MeOH (97:3) was applied to a Si gel (60 g)
column eluted with a gradient of \[ n \times \text{hexane–CH}_2\text{Cl}_2 \] (5:5
9:1). Fraction F-I eluted with CH$_2$Cl$_2$–MeOH (99:1) was
further resolved on another Si gel (40 g) column using the same
elution system as for fraction F-IX to yield seven tertiary
fractions (FIX-6–FIX-6-7). The activity was found in

4.3. Antagonism bioassays with $E$. gomezpompea against
endophytic fungi and some phytopathogenic microorganisms

Antagonism bioassays were performed against three
endophytic fungi that were also isolated from $C$. acuminata
(Verbenaceae) and four economically important phytopathogenic microorgan-
isms, two phytopathogenic fungi (Eumycota): Fusarium oxy-
sporum and Alternaria solani, and two phytopathogenic
fungus-like organisms (Oomycota): Phythophtora parasitica
and Phythophtora capsici. Nowadays, these fungoids oomycetes are classified within the phylum Heterokontophyta
(class Oomycota) which are primarily algae. The bioassays
were performed in 9 cm Petri dishes with PDA medium.
Because $E$. gomezpompea grows very slowly, inoculum
(5 mm$^3$ agar plug) was placed one side of the dish and grown
for 15 days at 28°C and a photoperiod of 12:12 with natural
light before the antagonism test. Inoculum of the respective
test fungus then was added to the other side of the dish.
The dish was sealed with Parafilm® and incubation was contin-
ued under the same conditions. Four replicate dishes were
used for each antagonism bioassay. Controls consisted of
each fungus growing individually under the same conditions.
Results of the antagonism tests were observed at different
times of incubation, depending on the growth rate of the test
microorganism; $A$. solani and $F$. oxysporum after 4 days of
incubation; $P$. capsici, $P$. parasitica, Colletotrichum sp. and
Phomopsis sp. after 38 days of incubation; and $G$. manguifera
after 10 days of incubation. The development of both micro-
organisms growing together was compared with the respec-
tive controls.

4.4. Cultivation

Fresh mycelium grown on potato-dextrose agar (PDA)
(Hanlin and Ulloa, 1988) medium at 28°C for 15 days
was inoculated into one 1000 ml Erlenmeyer flasks containing
500 ml potato-dextrose (PD) broth. After 15 days of
incubation at 28°C on rotary shaker at 200 rpm, this
culture was transferred as inoculum into fermentation equip-
ment. Fermentation was done on a 301 scale (PD medium)
in three M19-1400 fermenters, New Brunswick
scientific Co., Edison, NJ, USA). The fungus was grown at
28°C for 3 weeks with stirring at 200 rpm and an aera-
tion rate of 5 l/min.

4.5. Extraction and isolation of allelochemicals from
$E$. gomezpompea

At the end of fermentation, the culture medium was sep-
parated from the mycelium by filtration. The culture med-
ium (27 l) was concentrated at room temperature and
vacuum to 31 and was extracted three times with 31
of CH$_2$Cl$_2$ and then three times with 31 of EtOAc. Both
organic phases were combined and filtered over anhydrous
Na$_2$SO$_4$, and concentrated in vacuo to give a reddish solid
(830 mg). The mycelium was extracted the same way to
yield 14.25 g of a reddish solid.

The mycelial extract was subjected to silica gel CC
(120 g) eluting with a gradient of \[ n \times \text{hexane–CH}_2\text{Cl}_2–\text{MeOH} \] mixtures of increasing polarity. From this chromatogra-
phy, 175 fractions (100 ml each) were obtained. Thirty
fractions, F-I to F-XIII, were pooled according to TLC
analysis. Bioactivity in the antifungal bioassay showed nine
active pools: F-IV (2758 mg), F-V (239 mg), F-VI (72 mg),
F-VII (86 mg), F-VIII (204 mg), F-IX (1256 mg), F-X
(135 mg), F-XII (278 mg), and F-XIII (748 mg).

Active fraction F-IV, eluted with hexane–CH$_2$Cl$_2$ (5:5),
was resolved by preparative TLC (\[ n \times \text{hexane–CH}_2\text{Cl}_2 \] 2:8) to yield palmarumycin CP$_2$ (4) (25.4 mg) (Krohn et al.,
1994a,b; Singh et al., 1994; Ragot et al., 1999; Barrett et
al., 2002). From primary fraction V (eluted with \[ n \times \text{hexane–CH}_2\text{Cl}_2 \] 3:7), bioactive fraction F-I, eluted
with CH$_2$Cl$_2$–MeOH (99:1) was further resolved on another Si gel (40 g)
column using the same elution system as for fraction F-IX to yield seven tertiary
fractions (FIX-6–FIX-6-7). The activity was found in
tertiary fraction F-IX6-4 (77.5 mg), eluted with CH₂Cl₂. Finally, extensive TLC (CH₂Cl₂–MeOH; 98:2 × 2) of fraction F-IX6-4 yielded preussomerin EG₂ (2) (28.3 mg).

4.6. Bioassays with phytopathogenic and endophytic fungi

Organic extracts, fractions, and isolated compounds were tested for inhibitory effects on the growth of the seven test microorganisms. The extracts (culture and extracts were evaluated using a completely random design with four replications. Inoculated plates were incubated in darkness at 27 °C. The effects of treatments were determined by measuring the diameter growth of the mycelium after 3, 4 and 10 days of incubation depending on the growth speed of the test microorganism. Two perpendicular measurements of colony diameter were taken and the mean value was calculated. Results were analyzed by ANOVA and Tukey's statistical tests (Mead et al., 2002). IC₅₀ (inhibitory concentration for 50% diameter growth reduction) values for compound 1–3 and extracts were calculated by probit analysis based on the diameter growth of the mycelium bioassays.

4.7. Spectral data of compounds

4.7.1. Preussomerin EG₁ (1)

Yellow solid, mp 215.7 °C (decomp); [α]D −114 (CH₂Cl₂; c 1); UV (CH₂Cl₂) λ_max nm (log ε) 348 (3.10), 307 (2.72), 264 (3.29), 252 (3.24), 232 (3.66); IR ν_max (KBr) 3078, 1679, 1642, 1592, 1472, 1359, 1330, 1297, 1274, 1231 cm⁻¹; for 1H and 13C NMR spectroscopic data, see Tables 2 and 3; EIMS m/z 348 [M⁺ (81)], 303 (4), 191 (4), 175 (31), 174 (100), 173 (4), 147 (8), 146 (11), 118 (6) 75 (7); HRMS m/z 348.0631 (calcld for C₂₀H₁₄O₆, 348.0633).

4.7.2. Preussomerin EG₂ (2)

Yellow crystalline needles, mp 224 °C (decomp); [α]D −143 (MeOH; c 1); UV (MeOH) λ_max nm (log ε) 359 (3.10), 333 (2.94), 318 (3.04), 283 (2.63), 258 (3.66), 243 (3.49), 221 (3.95); CD (MeOH) Δε (nm) −1.0 × 10⁶ (219) −4.8 × 10⁷ (334); IR ν_max (KBr) 3452, 1692, 1648, 1596, 1471, 1329, 1291, 1226 cm⁻¹; for 1H and 13C NMR spectroscopic data, see Tables 2 and 3; EIMS m/z 366 [M⁺ (100)], 348 (7), 337 (18), 323 (6), 295 (6), 282 (12), 277 (6), 266 (6), 238 (13), 192 (23), 176 (20), 175 (10), 174 (11), 163 (9), 147 (6), 91 (8), 55 (8), 18 (26); HRMS m/z 366.0737 (calcld for C₂₀H₁₄O₆, 366.0739).

4.7.3. Preussomerin EG₃ (3)

Orange solid, mp 183.6–185.6 °C; [α]D −178 (MeOH; c 1); UV (MeOH) λ_max nm (log ε) 362 (3.71), 335 (3.17), 316 (3.31), 283 (3.04), 261 (3.89), 209 (2.53); CD (CHCl₃) Δε (nm) −7.3 × 10⁴ (334); IR ν_max (KBr) 3375, 2929, 2856, 1739, 1696, 1598, 1471, 1412, 1361, 1332, 1289, 1262, 1228 cm⁻¹; for 1H and 13C NMR spectroscopic data, see Tables 2 and 3; EIMS m/z 380 [M⁺ (100)], 365 (10), 347 (7), 321 (7), 294 (8), 293 (10), 277 (7), 266 (8), 265 (8), 238 (23), 210 (5), 192 (6), 189 (6), 175 (14), 174 (10), 147 (4), 119 (4), 91 (6), 75 (5), 55 (6); HRMS m/z 380.0892 (calcld for C₁₇₆H₁₆O₇, 380.0896).

4.7.4. Acetylation of preussomerin EG₁

Preussomerin EG₁ (1) (30 mg) was acetylated using Ac₂O (0.5 ml) in pyridine (0.5 ml), with work up as usual. The reaction mixture was purified by TLC (CH₂Cl₂–MeOH; 99:1) to yield monoacetoxy product (15 mg) and diacetylated derivative (12 mg). Monoacetoxypreussomerin EG₁ (1a); pale yellow solid, 1H NMR (CDCl₃, 500 MHz) 2.37 (3H, 3); OCOCH₃-9), 2.56 (1H, ddd, J = 13.5, 13.5, 5.5 Hz, H-2a), 2.73 (1H, ddd, J = 13.5, 5.5, 2.0 Hz, H-3b), 3.24 (1H, ddd, J = 19.0, 13.5, 5.5 Hz, H-2b), 6.58 (1H, d, J = 9.5 Hz, H-2), 7.03 (1H, d, J = 9.0 Hz, H-7), 7.05 (1H, dd, J = 8.0, 1.0 Hz, H-9), 7.08 (1H, d, J = 9.0 Hz, H-8), 7.18 (1H, d, J = 9.5 Hz, H-3), 7.55 (1H, dd, J = 8.0, 7.5 Hz, H-8), 7.61 (1H, dd, J = 7.5, 1.0 Hz, H-7); 13C NMR (CDCl₃, 125 MHz) 21.0 (OCOCH₃-9), 32.2 (C-3), 34.1 (C-2), 89.7 (C-4), 113.5 (C-10), 117.6 (C-5), 120.2 (C-12), 120.5 (C-7''), 121.0 (C-9''), 123.0 (C-8), 126.5 (C-7), 130.0 (C-8'), 130.4 (C-10'), 133.5 (C-2''), 141.0 (C-3''), 141.2 (C-6), 147.1 (C-9), 149.5 (C-6'), 169.8 (OCOCH₃-9), 184.0 (C-1'), 193.8 (C-1').

4.8. X-ray crystallographic analysis of palmarumycin CP₂ (4)

Empirical formula C₂₀H₁₄O₄, Mr = 318.31, 0.40 × 0.17 × 0.14 mm, colorless prism, from CH₂Cl₂ triclinic, P₁ (No. 2); a = 8.5182(6) Å, b = 12.7753(8) Å, c = 15.1233(10) Å, α = 65.4740(10)°, β = 79.9160(10)°.
\( \gamma = 86.8040(10)^{\circ}, \ V = 1473.87(17) \text{\AA}^3, \ Z = 4, \ D_{\text{cal}} = 1.435 \text{Mg/m}^3, \ m_{\text{p}} = 0.100 \text{mm}^{-1}, \ F(000) = 664, \ \text{Temp} = 298(2) K, \ \text{MoK\(\alpha \ \lambda = 0.71073 \text{\AA}, \ 1.50-25.00^\circ \ \text{of} \ \theta \ \text{collection,布鲁克智能短语APEX AXS CCD area detector, the data reduction was carried out with the SAINT program (Bruker, 2001), 12119 reflections were collected, of which 5176 (\(R_{\text{int}} = 0.0341) \) were independent reflections. The structure was solved and refined using full-matrix least-squares on \(F^2\) on the programs SHELXS-2006 (Sheldrick, 1997) and SHELXL-97 (Sheldrick, 1997), respectively. The program XSCHEL was used as an interface to the SHELX programs, and to prepare the figures. Two independent molecules were found in the unit cell. The final values of \(S = 0.899, \ R_I = 0.0373, \ \omega R_2 = 0.0787, \) were based on 5176 reflections observed with \(|I| > 2\sigma(I)|, 439 \) parameters, the largest diff. peak and hole were 0.143 and \(-0.183 \text{e A}^{-3}\).

5. Supplementary data

Crystallographic data have been deposited at the Cambridge Crystallographic Data Center (CCDC) (CCDC 651731).

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References


analogue of the fungal metabolite palmarumycin CPi. The Journal of Pharmacological and Experimental Therapeutics 296, 364–371.


