Cycloaspeptide A and Pseurotin A from the Endophytic Fungus
Penicillium janczewskii

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Penicillium janczewskii K. M. Zalessky was isolated as an endophytic fungus from the phloem of the Chilean gymnosperm Prumnopitys andina. When grown in liquid yeast extract-malt extract-glucose broth, the fungus produced two main secondary metabolites. The compounds were for the first time isolated from this species and identified by spectroscopic methods as pseurotin A and cycloaspeptide A. This is the first report on the production of cyclic peptides by endophytic fungi from Chilean gymnosperms. Pseurotin A and cycloaspeptide A presented low cytotoxicity towards human lung fibroblasts with IC_{50} values of 1000 μm. Pseurotin A showed a moderate effect against the phytopathogenic bacteria Erwinia carotovora and Pseudomonas syringae, with IC_{50} values of 220 and 112 μg ml^{-1}, respectively.

Key words: Penicillium janczewskii, Cycloaspeptide A, Pseurotin A, Endophyte

Introduction

Little is known about the metabolites produced by endophytic fungi of Chilean native plants, including gymnosperms (Hormazabal et al., 2005; Schmeda-Hirschmann et al., 2005).

Endophytic fungi are widespread in plants and represent a relevant component of plant communities (Gunatilaka, 2006; Owen and Hundley, 2004). The relation of the plant and fungus can range from symbiosis to parasitism, depending on the host-invader balance (Kogel et al., 2006). It has been shown that endophytes protect plants against pathogens and that host affinity is mediated by the leaf chemistry (Arnold et al., 2003). Those fungi have been recognized as a valuable source of novel bioactive metabolites including taxol, cryptocin, cryptocandin, jesterone, oocydin, isopestacin, the pseudomycins and ambuic acid (Strobel, 2002; Li et al., 1996) as well as podophyllotoxin (Puri et al., 2006) and volatile organic compounds (Strobel, 2006).

The metabolite production of endophytic fungi has been shown to be related with the genotypic diversity. In a comparative study of Cylindrocarpon destructans and Heliscus luguensis populations isolated from the roots of a single tree, Seymour et al. (2004) found a good correlation between genotype classification and the natural product patterns of the crude extracts obtained from the microbial cultures. The role of arbuscular mycorrhizal fungi and agricultural practices has been recently revised by Gosling et al. (2006).

Following our studies on bioactive products from endophytic fungi isolated from Chilean gymnosperms, we now report the secondary metabolites produced by Penicillium janczewskii obtained from the Podocarpaceae Prumnopitys andina (Poepp. ex Endl.) de Laub, known in Chile as “lleuque”.

Materials and Methods

Isolation and culture

The fungus was isolated from wood pieces collected on the western Andean slopes near Las Trancas, Chillán as described in a previous report (Schmeda-Hirschmann et al., 2005). The microorganism was identified by Prof. Eduardo Pontelli, Universidad Católica de Valparaíso; it is kept at the microbial strain collection of Universidad de Talca. Penicillium janczewskii was cultured in yeast extract-malt extract-glucose broth (YMG broth, 4 g yeast extract, 10 g malt extract, 10 g glu-
cose in 1 l water, pH 5.5) in Erlenmeyer flasks (14.4 l) at 25 °C under constant shaking (150 rpm). Once glucose in the medium was consumed (13 d), mycelium and culture broth were separated by filtration and extracted separately with EtOAc (3 ¥ 2 l). Some 640 mg of EtOAc-solubles were obtained from the culture filtrate and 2.15 g from the mycelium. The extract obtained from the culture medium was dissolved in MeOH and permeated in a Sephadex LH-20 column (length, 70 cm; internal diameter, 2 cm) with MeOH. Some 200 fractions of 4 ml each were collected and pooled together according to the TLC patterns. Fractions 56–59 (138 mg) were rechromatographed on silica gel with a petroleum ether (PE)/ethyl acetate (EtOAc)/EtOAc/acetone gradient. Some 190 fractions were collected and pooled together in 12 groups according to the TLC patterns. The pooled fractions 5 and 6 were purified by preparative TLC (silica gel, PE/EtOAc 3:7) affording 30 mg pseurotin A (1) and 10 mg cycloaspeptide A (2). The other fractions did not contained compounds of interest based on TLC analysis and NMR measurements and were not further investigated.

Compounds

Melting points were determined on a Koffler hot stage apparatus (Electrothermal 9100, Essex, UK) and are uncorrected. Optical rotations were obtained for solutions in CHCl₃ (concentrations expressed in g 100 ml⁻¹) on a Jasco DIP 370 polarimeter. IR spectra were recorded on a Nicolet Nexus FT-IR instrument. ¹H NMR spectra were recorded at 400 MHz and ¹³C NMR data were obtained at 100 MHz on a Bruker spectrometer (δ scale). TLC spots were visualized by spraying the chromatograms with H₂SO₄/EtOH (10:90) and heating at 110 °C for 3 min. Column chromatography was performed over Merck Kieselgel 60, particle size 0.063–0.200 mm. Mass spectra are presented as m/z (% rel. int.).


Cycloaspeptide A (2): Colourless resin. – EI-MS: m/z = 641 (29), 497 (45) [M-144], 421 (10) (497–76), 378 (7), 352 (19), 306 (9), 266 (20), 232 (9), 150 (34), 134 (100), 120 (31). – C₃₆H₄₃N₅O₆ (641). – FT-IR: ν = 3316, 1670, 1656, 1632, 1593, 1516, 1447, 753, 702 cm⁻¹. – ¹H and ¹³C NMR: see Table II.

Table I. ¹H and ¹³C NMR data of pseurotin A (1) (400 and 100 MHz, respectively; CDCl₃).

<table>
<thead>
<tr>
<th>H</th>
<th>δ_H</th>
<th>δ_C</th>
<th>HMBC</th>
</tr>
</thead>
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<tr>
<td>2</td>
<td>–</td>
<td>166.53  s</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>113.56  s</td>
<td>1.65</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>196.45  s</td>
<td>1.65</td>
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<tr>
<td>5</td>
<td>–</td>
<td>92.67   s</td>
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<td>6</td>
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<td>185.73  s</td>
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</tr>
<tr>
<td>7 NH</td>
<td>8.39 s</td>
<td>–</td>
<td>92.73</td>
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<tr>
<td>8</td>
<td>4.67 s</td>
<td>90.25   s</td>
<td>3.43</td>
</tr>
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<td>9</td>
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<td>73.15 d</td>
<td>8.39, 3.43</td>
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<td>10</td>
<td>4.73 dd (9, 4.4)</td>
<td>70.63 d</td>
<td>5.56, 4.73</td>
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<tr>
<td>11</td>
<td>5.25 br dd (11, 9)</td>
<td>126.50 d</td>
<td>2.05, 5.25</td>
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<tr>
<td>12</td>
<td>5.56 ddd (11, 7.8, 7.8)</td>
<td>137.04 d</td>
<td>0.96, 2.09</td>
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<tr>
<td>13</td>
<td>2.09 dq (7.8, 7.3)</td>
<td>21.43 t</td>
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<td>14.11 q</td>
<td>137, 21; 2.09</td>
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<td>15</td>
<td>1.65 s</td>
<td>6.00 q</td>
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<td>16</td>
<td>–</td>
<td>194.94  s</td>
<td>–</td>
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<td>17</td>
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<td>–</td>
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<td>18</td>
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<td>130.70  d</td>
<td>194, 134; 7.63</td>
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<tr>
<td>19, 23</td>
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<td>128.74 d</td>
<td>128, 132</td>
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<tr>
<td>20, 22</td>
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<td>134.76 d</td>
<td>130; 8.30, 7.63</td>
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<tr>
<td>21</td>
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<td>51.76 q</td>
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<td>OMe</td>
<td>3.43 s (3 H)</td>
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Table II. \textsuperscript{1}H and \textsuperscript{13}C NMR data of cycloaspeptide A (2) (400 and 100 MHz, respectively, CDCl\textsubscript{3}).

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<td>44.16 d</td>
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<td>16.22 q</td>
<td>44.16, 173.72</td>
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<td>CO</td>
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<td>173.93 s</td>
<td>0.40, 2.87</td>
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Phenylalanine

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<td>N-CH(_3)</td>
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<td>30.10 q</td>
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<td>63.37 d</td>
<td>30.10, 168.06</td>
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<td>C(\beta)</td>
<td>2.96 dd (13.0, 12.2), 3.48 dd (14.2, 4)</td>
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<td>C1</td>
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<td>137.61 s</td>
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<td>C2, C6</td>
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<td>129.40 d (2 C)</td>
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<tr>
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<td>129.18 d (2 C)</td>
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<tr>
<td>C4</td>
<td>7.26 m</td>
<td>134.33 d</td>
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<tr>
<td>CO</td>
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<td>169.63 s</td>
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Leucine

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<tbody>
<tr>
<td>NH</td>
<td>7.30 d (7.8)</td>
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<tr>
<td>C(\alpha)</td>
<td>4.72 ddd (8, 8, 5), 1.86 ddd (13.0, 8.5, 5.5)</td>
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<td>C(\beta)</td>
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<td>C(\gamma)</td>
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<td>24.88 d</td>
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<td>CH(_3)</td>
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<td>CH(_2)</td>
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Tyrosine

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<td>C(\alpha)</td>
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<tr>
<td>C1</td>
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<td>130.27 d (2 C)</td>
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<td>C4</td>
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<td>168.19 s</td>
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\textit{o}-Aminobenzoic acid

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<tr>
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<td>–</td>
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<tr>
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<td>115.04 s</td>
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<tr>
<td>C2</td>
<td></td>
<td>141.52 s</td>
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</tr>
<tr>
<td>C3</td>
<td>7.44 dbr (7.8)</td>
<td>126.92 d</td>
<td></td>
</tr>
<tr>
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<td>8.86 d (8.6)</td>
<td>120.90 d</td>
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<tr>
<td>CO</td>
<td></td>
<td>168.06 s</td>
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\textit{Antibacterial and antifungal activity}

Pseurotin A was assessed for antimicrobial activity by the agar diffusion method at a content of 50 \textit{µg} disk\textsuperscript{-1} towards the following microorganisms: Gram-positive bacteria: \textit{Bacillus brevis} (local isolate), \textit{Bacillus subtilis} (ATCC 6633) and \textit{Micrococcus luteus} (ATCC 381); Gram-negative bacteria: \textit{Enterobacter dissolvens} (LMG 2683); fungi: \textit{Mucor miehei} (Cooney et Emerson Tü 284), \textit{Paeclomycyes variotti} (ETH 44646) and \textit{Penicillium notatum} (Collection University of Kaiserslautern, Germany). The activity of the extracts was estimated by the growth inhibition (in mm) as follows: < 8 mm, inactive; 8–12 mm, weak activity; 13–15 mm, moderate activity; > 15 mm, strong activity. As references, the commercial antifungal benlate [benomyl, methyl-1-(butylcarbamoyl)-2-benzimidazole carbamate, 50\% active ingredient, 50 \textit{µg} benomyl disk\textsuperscript{-1}] and chloramphenicol (30 \textit{µg} disk\textsuperscript{-1}) were used.

The antifungal activity of pseurotin A against \textit{Alternaria alternata} (Centro Micológico, Univer-
Cytotoxicity

The cytotoxic effect of the compounds, expressed as cell viability, was assessed on the permanent cell line derived from human lung fibroblasts (MRC-5) (ATCC Nr CCL-171). MRC-5 fibroblasts were grown as monolayers in minimum essential Eagle medium (MEM), with Earle’s salts, 2 mM L-glutamine and 1.5 g l⁻¹ sodium bicarbonate, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin in a humidified incubator with 5% CO₂ in air at 37 °C. Cells were seeded at a density of 2.5 · 10³ cells per well in 96-well plates. Confluent cultures were treated with medium containing the compounds at concentrations ranging from 3.8 up to 1000 μM. The substance was firstly dissolved in DMSO and then in the medium supplemented with 2% FBS. The final content of DMSO in the test medium and controls was 1%. Cells were exposed for 24 h to the test medium with or without the compound (control). Each drug concentration was tested in quadruplicate, and repeated three times in separate experiments. At the end of the incubation, the neutral red uptake (NRU) assay was carried out as described by Rodríguez and Haun (1999). To calculate the IC₅₀ values the results were transformed to percentage of controls, and the IC₅₀ values were graphically obtained from the dose-response curves.

Results and Discussion

From the YMG broth culture of *Penicillium janczewskii* K. M. Zalessky, isolated from the phloem of the Chilean native gymnosperm *Prumnopitys andina*, two compounds were isolated and identified as pseurotin A (1) and cycloaspeptide A (2) by spectroscopic means (Fig. 1).

At a content of 50 μg disk⁻¹, pseurotin A was inactive against *Enterobacter dissolvens*, *Micrococcus luteus* and *Penicillium notatum* but showed a weak effect on *Bacillus brevis*, *B. subtilis*, *Mucor miehei* and *Paecilomyces variotti* with growth inhibition of 8, 8, 7 and 9 mm, respectively. The antifungal effect of compound 1 against *Botrytis cinerea* and *Alternaria alternata* was low (Table III). In the antibacterial assay against the phytopathogenic bacteria *Erwinia carotovora* and *Pseudomonas syringae*, pseurotin A presented a moderate activity (Table III), with IC₅₀ values of 220 and 112 μg ml⁻¹, respectively. The effect of pseurotin A on *P. syringae* was close to the value observed for penicillin G, but much higher than that of streptomycin.

The cytotoxicity of pseurotin A (1) towards human lung fibroblasts was low, with an IC₅₀ value of 1000 μM while the IC₅₀ value of cycloaspeptide A (2) was > 1000 μM.
Pseurotin A has been previously reported from *Pseudeurotium ovalis* (Bloch and Tamm 1981; Breitenstein et al., 1981) and *Aspergillus fumigatus* as well as from *Pochonia chlamydosporia* var. *cate- 

Three natural products of different structure have been reported for *P. janczewskii*, including *P. janczewskii* grown on liquid potato-dextrose medium was reported (Schmeda-Hirschmann et al., 2005). The chemical diversity of the compounds produced by the same species either isolated from different natural sources or grown in different media indicates the high versatility of the fungus in producing natural products and shows the potential of new research work on endophytic microorganisms when looking for bioactive products. The potential of this approach when looking for bioactive fungal metabolites has been outlined by Gunatilaka (2006) in a recent review. A related cyclic peptide was produced by a fungal strain obtained from the seed of *Avicennia marina*.

According to Madi and Katan (1998), when the filtrate of a liquid culture of *Penicillium janczew- 

Several natural products of different structure have been reported for *P. janczewskii*, including *cis*-fumagillin (Kwon et al., 2000), quinolinone alkaloids (He et al., 2005), peniprequinolone and gli- 

Table III. Antimicrobial activity (μg ml⁻¹) of pseurotin A and cycloaspeptide A determined by the microdilu- 

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<tr>
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<tbody>
<tr>
<td>Pseurotin A</td>
<td>220</td>
<td>112</td>
<td>&gt;250</td>
<td>125</td>
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<tr>
<td>Cycloaspeptide A</td>
<td>–</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td>–</td>
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<td>Streptomycin</td>
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<td>Penicillin G</td>
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<td>Iprodione</td>
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<td>15.6</td>
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E. c., *Erwinia carotovora*; P. s., *Pseudomonas syringae*; B. c., *Botrytis cinerea*; A. a., *Alternaria alternata*; –, not done.

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