

## CR377, a New Pentaketide Antifungal Agent Isolated from an Endophytic Fungus

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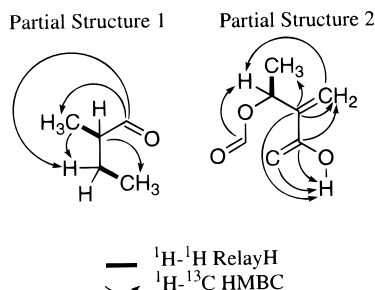
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Cultures of endophytic fungi collected in the Guanacaste Conservation Area of Costa Rica were screened for antifungal activity. CR377, a new pentaketide antifungal agent, was isolated from the culture broth of a fungus, CR377 (*Fusarium* sp.), that showed potent activity against *Candida albicans* in this assay. The structure of CR377 was established using 1- and 2-D NMR and HRFABMS.

Filamentous fungi have traditionally been a very fertile source of natural products, many of which have been useful prototypes for pharmaceutical agents.<sup>1–5</sup> Endophytic fungi, fungi including filamentous fungi that grow inside vascular plants, now provide the opportunity to greatly expand the known fungal biochemical diversity, as current estimates suggest  $1.3 \times 10^6$  unique endophytes live in the 270 000 known vascular plants.<sup>6,7</sup> The ease with which this diverse group of fungi can be harvested and cultured has made them a valuable resource in several laboratories' continuing efforts to characterize novel biologically active natural products and, more specifically, new antifungal agents. The need for new antifungal agents is highlighted by the fact that all major antifungals now available function by one of only four methods: inhibition of macromolecular synthesis, impaired membrane barrier function, inhibition of ergosterol synthesis, or disruption of microtubules.<sup>8</sup> We assayed agar plugs from a library of endophytes collected in the Guanacaste Conservation Area in Costa Rica for activity against fungal pathogens and identified a fungus, CR377, that showed potent anti-*Candida albicans* activity. CR377, *Fusarium* sp., was isolated from the interior of a surface-sterilized piece of *Selaginella pallescens* stem tissue. In this paper we report the isolation and characterization by HRFABMS and NMR of CR377 (**1**), a new pentaketide antifungal agent.

The compound responsible for the antifungal activity produced by CR377 was pursued when ethyl acetate extracts of large-scale potato dextrose broth (PDB) grown cultures displayed the same potent antifungal activity initially observed in the agar plug screen. CR377 (**1**) was purified to homogeneity by normal phase methods from the hexane extract of 21-day-old fungal cultures grown in PDB. HRFABMS indicated  $C_{12}H_{16}O_4$  (HRMS-FAB ( $m/z$ ):  $[M + H]^+$  calcd for  $C_{12}H_{17}O_4$  225.1127; found, 225.1125) as the molecular formula. The presence of three methyl groups, one exocyclic double bond, one methylene, two methines, and one strongly deshielded exchangeable proton was deduced from  $^1H$  and  $^1H-^{13}C$  DEPT NMR experiments. The  $^{13}C$  NMR spectrum suggested the presence of an additional double bond and two carbonyl carbons, a ketone ( $\delta$  211.8) and an ester ( $\delta$  163.5).

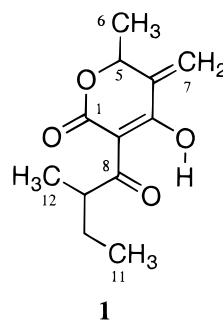
Three two-carbon spin systems were defined by  $^1H-^1H$  RelayH experiments (Figure 1). Two of these systems, C-9, C-12 and C-10, C-11, are linked by a long-range  $^1H-^{13}C$  HMBC correlation from the C-12 methyl to the methylene protons of C-10. An additional HMBC correlation from the



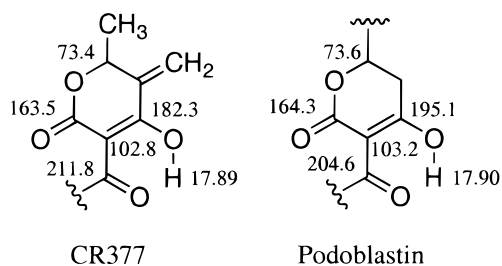
**Figure 1.**  $^1H-^1H$  RelayH and  $^1H-^{13}C$  HMBC correlations used to determine the structure of CR377.

C-8 carbonyl to the C-12 methyl protons confirmed partial structure 1 (Figure 1). Additional long-range correlations involving the third two-carbon spin system (C-5, C-6) define partial structure 2. The C-5, C-6 spin system is allylic to the C-4, C-7 exomethylene, as shown by HMBC correlations from C-4 to the C-6 methyl protons and C-7 to H-5. The C-2, C-3 enol, suggested by long-range correlations from both C-2 and C-3 to the highly deshielded exchangeable proton at  $\delta$  17.89, is linked to the C-4, C-7 double bond by HMBC correlations from C-4 to H-3 and from C-3 to both H-7 methylene protons. The presence of an HMBC correlation from the C-1 ester to the deshielded H-5 ( $\delta$  5.04) but not the C-6 methyl protons suggests that partial structure 2 is completed by linking the C-1 ester to C-5 through the ester oxygen (Figure 1).

Partial structures 1 and 2 contain all of the atoms that are predicted by HRFABMS to be present in CR377. On the basis of the unsaturation index of 5 for the molecular formula  $C_{12}H_{16}O_4$ , one unsaturation must still be incorporated into the final structure. This can only be achieved, using no additional atoms, by closing the lactone and connecting the two partial structures to give CR377 (**1**), a 2-methylbutyraldehyde-substituted  $\alpha$ -pyrone containing an exocyclic methylene. The presence of the  $\beta$ -tricarboxyl in



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**Figure 2.** NMR chemical shift comparison of the  $\beta$ -tricarbonyl of CR377 with the corresponding region of podoblastin.

the final CR377 (**1**) structure is supported by the comparison of  $^{13}\text{C}$  and  $^1\text{H}$  chemical shifts for this region with those reported for this moiety in other natural products: dehydroacetic acid,<sup>9</sup> alternaric acid,<sup>10–12</sup> and podoblastin<sup>13</sup> (Figure 2).

Dehydrocarolic acid and the agglomerin<sup>14</sup> type antibiotics contain five-membered  $\alpha$ -pyrones with exocyclic methylenes. However, no six-membered  $\alpha$ -pyrones<sup>9–15</sup> possessing this unique exocyclic alkene have been described. Although CR377 (**1**) is most likely derived from a doubly methylated (C-7 and C-12) pentaketide precursor, it is not possible to identify a continuous pentaketide chain that could lead to the final structure. CR377 appears instead to have arisen from the condensation of two independent di- and triketide fragments (C-8–11 and C-1–6 or C-3–6 and C-1, 2, 8–11), as shown for the related alternaric acid polyketides.<sup>10</sup> The condensation of either pair of fragments would lead to an appropriate pentaketide precursor for CR377.

CR377 (**1**) shows potent antifungal activity against strains of *Candida albicans* in agar diffusion assays performed on fungal lawns. Thirty micrograms of CR377 and 100 units of a nystatin control (approximately 30  $\mu\text{g}$ ) produce similar zones of growth inhibition against both *Candida albicans* strains *wisconsin* and *109*. CR377 produces inhibition zones of 20 and 24 mm, and nystatin results in zones of 19 and 22 mm against the *wisconsin* and *109* strains, respectively. Interestingly, CR377 does not inhibit the growth of *Saccharomyces cerevisiae* at 30  $\mu\text{g}$ , while nystatin produced a 21 mm zone of growth inhibition.

The ease with which large numbers of culturable endophytic fungi can be harvested from plants has made it possible to create a large, easily screened library of fungal extracts. These libraries of endophytic fungal extracts are good sources for the continued discovery of structurally unique bioactive natural products.

## Experimental Section

**General Experimental Procedures.**  $^{13}\text{C}$  and  $^1\text{H}$ - $^{13}\text{C}$  DEPT NMR spectra were measured using a 400 MHz Varian Unity spectrometer.  $^1\text{H}$ ,  $^1\text{H}$ - $^1\text{H}$  RelayH,  $^1\text{H}$ - $^{13}\text{C}$  HMQC, and  $^1\text{H}$ - $^{13}\text{C}$  HMBC NMR spectra were measured using a 500 MHz Varian Unity spectrometer.  $^{13}\text{C}$  and  $^1\text{H}$  chemical shifts were referenced with the  $\text{CD}_2\text{Cl}_2$  solvent peaks at  $\delta$  53.8 and  $\delta$  5.32, respectively. The UV spectrum was measured using a Shimadzu UV-160A spectrophotometer. The IR spectrum was recorded on a Perkin-Elmer 16PC FTIR spectrometer. Optical rotation data was obtained using a Perkin-Elmer 241 polarimeter. All mass spectra were run by the University of Illinois Mass Spectrometry facility. HPLC analysis was performed on a Hewlett-Packard 1050 using a Supelco semi-prep C-18 column (25 cm  $\times$  10 mm).

**Fungal Material.** CR377 was isolated from the interior of a *Selaginella pallescens* stem harvested from the Guanacaste Conservation Area in Costa Rica. *S. pallescens* plant tissue was surface sterilized by successive 5-min washes in 10%

bleach, 70% EtOH, and sterile  $\text{H}_2\text{O}$  (3 $\times$ ). Five millimeter squares of plant tissue were cut from the surface-sterilized specimen and placed on PDA plates to promote fungal growth. CR377 was subcultured from the fungi that grew from these samples after 2 days and then successively subcultured to obtain a pure fungal culture. The taxonomic identification of CR377 as *Fusarium* sp. was performed by Analytical Services, Inc. (Williston, VT). A voucher specimen of the culture, CR377, has been submitted to the Cornell University fungal herbarium (CUP).

**Culture Conditions, Extraction, and Isolation.** The fungus was grown in 1 L flasks containing 500 mL of PDB for 21 days with continuous shaking. The 21-day-old cultures were extracted with hexanes to give an antifungal-active crude extract. The methylene chloride-soluble material from this extract was applied to a silica column and eluted with 100%  $\text{CH}_2\text{Cl}_2$  to give a colorless oil possessing all of the antifungal activity. This material was found to be a single pure compound (**1**) by normal phase TLC, reversed phase HPLC (70:30 acetonitrile- $\text{H}_2\text{O}$ , 2 mL/min,  $t_{\text{R}}$  = 12.5 min), and NMR.

**CR377 (1):** colorless oil,  $[\alpha]_{\text{D}}^{25} +21.8^\circ$  (c 1.0,  $\text{CH}_2\text{Cl}_2$ ); UV ( $\text{CH}_2\text{Cl}_2$ )  $\lambda_{\text{max}}$  230, 300 nm; IR (NaCl, thin film)  $\nu_{\text{max}}$  1716, 1646, 1558, 1458, 1056  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{CD}_2\text{Cl}_2$ , 500 MHz)  $\delta$  17.89 (1H, s, OH), 6.33 (1H, d, 2.0, H-7), 5.74 (1H, d, 2.0, H-7), 5.04 (1H, tq, 2.0, 6.0, H-5), 3.82 (1H, s, 6.5, 7.0, H-9), 1.75 (1H, m, H-10), 1.57 (3H, d, 6.0, H-6), 1.44 (1H, m, H-10), 1.15 (3H, d, 7, H-12), 0.90 (3H, t, 7, H-11);  $^{13}\text{C}$  NMR ( $\text{CD}_2\text{Cl}_2$ , 100 MHz)  $\delta$  211.8 (C-8) 182.3 (C-3), 163.5 (C-1), 139.1 (C-4), 122.9 (C-7), 102.8 (C-2), 73.4 (C-5), 43.4 (C-9) 27.6 (C-10), 19.6 (C-6), 16.8 (C-12), 12.1 (C-11); HMBC C-1 (H-5), C-2 (OH), C-3 (H-5, -7, OH), C-4 (H-5, -6, -7, OH), C-5 (H-6, -7), C-6 (H-5), C-7 (H-5), C-8 (H-9, -10, -12), C-9 (H10, -11, -12), C-10 (H-9, -11, -12), C-11 (H-10), C-12 (H-9, -10); HRMS-FAB ( $m/z$ )  $[\text{M} + \text{H}]^+$  calcd for  $\text{C}_{12}\text{H}_{17}\text{O}_4$  225.1127; found, 225.1125.

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**Supporting Information Available:**  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra for CR377. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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