Isolation, Optimization of Production and Structure-Activity Relationship Studies of Monocillin I, the Cytotoxic Constituent of Paraphaeosphaeria quadriseptata

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In our continuing search for anticancer agents from the rhizosphere microflora of Sonoran desert plants1–3, an EtOAc extract of Paraphaeosphaeria quadriseptata, a fungus inhabiting the rhizosphere of the Christmas cactus, Opuntia leptocaulis DC. (Cactaceae), exhibited significant cytotoxicity against several human cancer cell lines. Cytotoxicity-guided fractionation afforded monocillin I (1) as the only active compound of this extract. Monocillin I has previously been isolated from the fungus Monocillium nordinii4,5, and the related resorcylic acid lactones, radicicol (monorden A) (2) and monocillins II–V have been encountered in Cylindrocarpon radicicola6, Penicillium luteo-aurantium7, Diheterosporia chlamydosporia8, Pochonia chlamydosporia var. catenulata9, Humicola fuscoatra NRRL 2298010, and Humicola sp. F0-294211,12. Although terrestrial fungi of the genus Paraphaeosphaeria have not been investigated previously for secondary metabolites, two 10-membered macrolides, modiolides A and B, and a linear pentaketide, modiolin, have been recently encountered in a marine-derived Paraphaeosphaeria sp. N-11913. In this report, we briefly describe the isolation of monocillin I (1) from P. quadriseptata, our studies on optimization of its production, and cytotoxic activities of 1 and its structural analogs 3–6 obtained by chemical derivatization of 1.

Materials and Methods

General Experimental Procedures
Physical and Spectral Data
Melting points were determined on a Gallenkamp micromelting point apparatus and are uncorrected. IR spectra for KBr disks were recorded on a Shimadzu FTIR-
Isolation and Identification of the Fungus

Excised roots (1 cm long sections; ca. 5 g) of the Christmas cactus (O. leptocaulis) collected at Tumamoc Hills in Tucson, Arizona, U.S.A., were placed in 5 ml phosphate buffered saline (PBS, 0.1 M, pH = 7.4) and microorganisms were detached from the roots by vortexing and sonication. Serial dilutions of the suspension were plated onto potato dextrose agar (PDA, Difco, Plymouth, MN) supplemented with chloramphenicol and streptomycin. After 4 days of incubation at 25 °C, single colonies were transferred on to water agar containing the same antibiotics and after 3 days a pure culture of the fungus was obtained by hyphal tipping. It was identified by Ms. DONNA BIGELOW (Department of Plant Sciences, University of Arizona) as Paraphaeosphaeria quadriseptata by analysis of the ITS regions of the ribosomal DNA as described previously. The strain is deposited in the Division of Plant Pathology and Microbiology, Department of Plant Sciences and Southwest Center for Natural Products Research and Commercialization of the University of Arizona microbial culture collection under the accession number AH-45-00-F20. The organism was subcultured using Petri dishes with PDA and for long term storage isolates were subcultured on PDA slants, overlaid with 40% glycerol and stored at -80 °C.

Cultivation in Solid Media, Extraction and Isolation of Monocillin I

For isolation of bioactive compounds the fungus was cultured in forty 800 ml T-flasks each containing 135 ml of PDA coated on five sides of the T-flask (total surface area ca. 460 cm²). After 28 days at 27 °C, MeOH (200 ml) was added to each of the forty T-flasks, allowed to soak overnight at room temperature and the resulting extract was filtered through Whatman No. 1 filter paper and a layer of Celite 545. The filtrate was concentrated to one-fourth of its original volume and extracted with EtOAc (5 × 500 ml). Evaporation under reduced pressure afforded an EtOAc extract (1.85 g), which was found to be cytotoxic (at 10 μg/ml). The cytotoxic EtOAc extract (1.80 g) was partitioned between hexane and 80% aqueous MeOH and the cytotoxic aqueous MeOH fraction was diluted to 60% aqueous MeOH by the addition of water and extracted with CHCl₃. Evaporation of CHCl₃ under reduced pressure yielded a pale brown semi-solid (1.31 g) which was found to be cytotoxic. A portion (1.30 g) of this was subjected to gel permeation chromatography on a column of Sephadex LH-20 (40.0 g) made up in hexane: CH₂Cl₂ (1:4) and eluted with hexane: CH₂Cl₂ (1:4) (700 ml), CH₂Cl₂: acetone (3:2) (250 ml), CH₂Cl₂: acetone (1:4) (250 ml), and finally with MeOH (250 ml). Twenty four fractions (50 ml each) were collected (F1 ~ F24) of which fractions F7 ~ F17 were found to be cytotoxic. Fractions F7 ~ F17 were combined and further fractionated on silica gel (13.0 g) by elution with CH₂Cl₂ followed by increasing amounts of MeOH in CH₂Cl₂. Fractions eluted with 0.5% MeOH in CH₂Cl₂ were found to be cytotoxic and these fractions were combined and evaporated to yield monocillin I (1) (521 mg).

Monocillin I (1): White solid; mp 129 ~ 132 °C; [α]D270 = -32.4° (c 1.0, CHCl₃); physical and spectral data (¹H and ¹³C NMR) were consistent with literature values.

Cultivation in Liquid Media and Extraction

A slant culture of P. quadriseptata grown on PDA was used to inoculate 500-ml Erlenmeyer flasks containing 100 ml each of the following four media: (A) PDB prepared with distilled water; (B) PDB prepared with tap water; (C) synthetic medium containing corn steep liquor (4.0 g), glucose (2.0 g), yeast extract (1.0 g) in distilled water (1000 ml); (D) synthetic medium containing corn steep liquor (5.0 g), KH₂PO₄ (1.5 g), MgSO₄ (0.5 g), malt extract (1.0 g), yeast extract (2.0 g) in distilled water (1000 ml). Flasks were shaken on a rotary shaker at 27 °C and 160 rpm. On days 10 and 15, aliquots of culture medium (25 ml) were withdrawn from each flask, neutralized to pH 7 with NaOH or HCl as appropriate and each extracted with 3 × 25 ml of EtOAc. After 20 days the remaining volume (50 ml) of each of the cultures was extracted with 3 × 50 ml of EtOAc. The EtOAc extracts were separately evaporated, dried under reduced pressure, and the weights were recorded. The yield of each EtOAc extract/1000 ml of culture (average of 3 experiments) is presented graphically in Fig. 1.

HPLC Analysis of Extracts for Monocillin I

For determination of the amount of monocillin I (1) in culture broths, each of the above EtOAc extracts was dissolved in MeOH and analyzed by LC-UV (LC-10 AD equipped with SCL-10A system controller, SIL-10AD auto injector and SPD-M10A diode array detector, Shimadzu) under the following conditions: Watersorb-MV C8 5 μm column (5 × 250 mm), 40 minutes linear gradient from 50%
MeOH - water (0.5% HCOOH in water) to 100% methanol, 1.0ml/minute. Monocillin I was eluted as a peak with the retention time 13.55 minutes. A typical HPLC trace obtained is shown in Fig. 2 with peak 6 corresponding to monocillin I (1). The percentage of monocillin I in each extract was obtained from the peak area and was used to calculate the amount of 1 produced by 1000ml of each culture (Fig. 1).

Methylation of Monocillin I

Methyl iodide (200µl) and K₂CO₃ (20mg) were added to a solution of monocillin I (5.0mg) in acetone (500µl) and stirred at 23°C. The reaction was monitored by TLC. After the disappearance of the starting material (30 minutes), the reaction mixture was filtered and the filtrate was evaporated under reduced pressure to obtain the crude product which was separated on preparative TLC (silica gel) using CH₂Cl₂ (triple elution) to yield 3 (3.9mg) and 4 (1.2mg).

Monomethyl Monocillin I (3): Colorless semisolid, [α]₂⁰° -28.0° (c 1.0, MeOH); UV (MeOH) λₘₚₖ (log ε) 261.8 (4.72), 214.8 (4.96); ¹H NMR (CDCl₃, 600MHz) 11.40 (1H, s, OH), 7.78 (dd, J=16.0, 11.0 Hz, H-4'), 6.36 (1H, d, J=2.5 Hz, H-3), 6.35 (1H, dd, J=2.5, 0.5 Hz, H-5), 6.23 (1H, td, J=11.0, 1.0 Hz, H-5'), 5.99 (1H, d, J=16.0 Hz, H-3'), 5.89 (1H, dd, J=11.0, 2.5 Hz, H-6'), 5.55 (1H, m, H-10'), 5.16 (1H, d, J=14.0, H-1'a), 3.74 (3H, s, OMe), 3.59 (1H, d, J=14.0 Hz, H-1'b), 3.26 (1H, d, J=2.5 Hz, H-7'), 3.04 (3H, s, OMe), 2.68 (1H, d, J=7.0 Hz, H-8'), 2.63 (1H, d, J=7.0 Hz, H-9'), 2.53 (1H, d, J=10.0 Hz, H-10'), 2.30 (1H, d, J=11.0 Hz, H-9'), 1.83 (1H, d, J=8.0 Hz, H-8'), 1.59 (1H, d, J=10.0 Hz, H-10'), 1.27 (3H, s, OMe), 1.08 (1H, d, J=7.0 Hz, H-9'), 1.03 (1H, d, J=8.0 Hz, H-8'), 1.01 (1H, d, J=9.0 Hz, H-7'), 0.88 (3H, s, OMe), 0.86 (1H, d, J=7.0 Hz, H-10'), 0.83 (1H, d, J=9.0 Hz, H-7'), 0.79 (1H, d, J=10.0 Hz, H-8'), 0.76 (1H, d, J=11.0 Hz, H-9'), 0.74 (3H, s, OMe), 0.71 (1H, d, J=7.0 Hz, H-8'), 0.69 (1H, d, J=9.0 Hz, H-7'), 0.65 (1H, d, J=10.0 Hz, H-8').
3.06 (1H, dt, J=9.5, 2.5 Hz, H-8′), 2.40 (dt, J=15.1, 3.0 Hz, H-9’a), 1.60 (3H, d, J=6.9 Hz, CH3); APCIMS +ve mode m/z 345 [M+H]+; APCIMS –ve mode m/z 343 [M-H]+; HRFAB-MS: calcld. for C19H21O6 [M+H]+ 345.1332; found: m/z 345.1344.

Dimethyl Monocillin I (4): Colorless semisolid, [α]D27 -32.9° (c 1.0, MeOH); UV (MeOH) λmax (log ε) 277.6 (5.13), 203.8 (5.49); 1H NMR (CDCl3, 600MHz) 7.66 (dd, J=15.9, 10.6 Hz, H-4′), 6.33 (2H, s, H-3 and H-5), 6.21 (1H, t, J=10.6 Hz, H-5′), 5.99 (1H, d, J=15.9 Hz, H-3′), 5.79 (1H, dd, J=10.6, 4.5 Hz, H-6′), 5.33 (1H, m, H-10′), 3.93 (1H, d, J=13.7 Hz, H-1’a), 3.83 (1H, d, J=13.7 Hz, H-1’b), 3.77 (3H, s, OMe), 3.74 (3H, s, OMe), 3.54 (1H, dd, J=4.5, 2.0 Hz, H-7’), 3.06 (1H, ddd, J=4.0, 3.5, 2.0 Hz, H-8’), 2.43 (dt, J=15.0, 4.0 Hz, H-9’a), 1.70 (1H, ddd, J=15.0, 7.5, 3.5 Hz, H-9′b), 1.52 (3H, d, J=6.4 Hz, CH3); APCIMS +ve mode m/z 359 [M+H]+; APCIMS –ve mode 357 [M-H]+.

Catalytic Hydrogenation of Monocillin I
To a solution of monocillin I (3.5mg) in EtOH (0.3ml) was added 10% Pd on C (1.0mg) and stirred under an atmosphere of H2. After stirring for 5 minutes the reaction mixture was filtered, evaporated under reduced pressure and separated on preparative TLC (silica gel) using 6% MeOH in CH2Cl2 as eluant to give tetrahydromonocillin I (5) (2.1mg) and hexahydromonocillin I (6) (1.2mg).

Tetrahydromonocillin I (Monocillin V) (5): Colorless gum, [α]D27 -18.3° (c 1.0, CHCl3); UV (MeOH) λmax (log ε) 304.2 (4.58), 265.4 (4.85), 215.4 (5.19), 201.2 (5.09); 1H NMR (CDCl3, 600MHz) δ 11.64 (1H, s, OH), 6.35 (1H, d, J=2.5 Hz, H-3), 6.16 (1H, d, J=2.5 Hz, H-5), 5.26 (1H, m, H-10′), 4.36 (1H, d, J=17.2 Hz, H-1′a), 3.61 (1H, d, J=17.2 Hz, H-1′b), 2.81 (1H, dt, J=5.4, 2.9 Hz, H-8′), 2.65 (1H, ddt, J=9.2, 2.5 Hz, H-7′), 2.45 (2H, brt, J=6.6 Hz, H-3′ab), 2.21 (1H, ddd, J=15.8, 6.5, 3.6 Hz, H-9′a), 2.08 (1H, ddd, J=14.1, 6.9, 3.2 Hz, H-6′a), 1.96 (1H, ddd, J=15.8, 5.8, 2.3 Hz, H-9′b), 1.71 (1H, m, H-4′a), 1.62–1.50 (3H, m, H-4′b, H-5′a, H-5′b), 1.44 (3H, d, J=6.6 Hz, CH3), 1.11 (1H, m, H-6′b); 13C NMR (CDCl3, 125MHz) δ 208.9 (C, C-2′), 170.2 (C, C-7′), 163.9 (C, C-4), 160.8 (C, C-2), 139.1 (C, C-6), 112.9 (CH, C-5), 106.2 (C, C-1), 103.1 (CH, C-3), 70.55 (CH, C-8′), 50.0 (CH, C-1′), 42.7 (CH2, C-9′), 40.7 (CH2, C-3′), 34.4 (CH2, C-7′), 25.2 (CH2, C-5′), 22.9 (CH2, C-6′), 21.4 (CH2, C-4′), 19.4 (CH2, C-11′); APCIMS +ve mode m/z 335 [M+H]+; APCIMS –ve mode 333 [M-H]+.

Hexahydromonocillin I (6): Colorless gum, [α]D27 -20.9° (c 1.0, CHCl3); UV (MeOH) λmax (log ε) 299.4 (4.54), 263.2 (4.82), 214.2 (5.26), 201.2 (5.24); 1H NMR (CDCl3, 600 MHz) δ 11.40 (1H, s, OH), 6.32 (1H, d, J=2.5 Hz, H-3), 6.17 (1H, d, J=2.5 Hz, H-5), 5.47 (1H, m, H-10′), 4.22 (1H, d, J=17.0 Hz, H-1′a), 3.96 (1H, d, J=17.0 Hz, H-1′b), 3.72 (1H, m, H-8′), 2.52 (1H, ddd, J=15.7, 8.1, 4.2 Hz, H-3′a), 2.22 (1H, ddd, J=15.7, 8.5, 4.8 Hz, H-3′b), 1.98 (1H, ddd, J=14.8, 8.2, 4.3 Hz, H-9′a), 1.85 (1H, ddd, J=14.8, 7.5, 2.9 Hz, H-9′b), 1.71–1.59 (3H, m, H-4′a, H-4′b, H-5′a), 1.52–1.47 (2H, m, H-5′b, H-6′a), 1.43 (3H, d, J=6.6 Hz, CH3); APCIMS +ve mode m/z 337 [M+H]+; APCIMS –ve mode 335 [M-H]+.

Cytoxicity Bioassays
A tetrazolium-based colorometric assay (MTT assay)14) was used to evaluate the in vitro cytotoxicity of compounds against human non-small cell lung carcinoma (NCI-H460), human breast carcinoma (MCF-7), human glioma (SF-268), human pancreatic cancer (MIA Pa Ca-2), and human normal fibroblast (WI-38) cells as previously reported1.

Results and Discussion
Bioassay-guided fractionation of the cytotoxic EtOAc extract of P. quadriseptata cultured in PDA (solid medium) yielded monocillin I (1) (96.5 mg/1000 ml of PDA) as the only cytotoxic constituent. Monocillin I showed selective cytotoxicity against the human cancer cell lines (NCI-H460, MCF-7, SF-268, and MIA Pa Ca-2) compared with the normal human fibroblast cell line, WI-38 (Table 1). This selective cytotoxicity of 1 together with the reported biological activities and clinical potential of its closely related analog, radicicol (2)15,16) prompted us to subject monocillin I and its analogs to more extensive in vitro and in vivo biological evaluation which required the development of an efficient method for large-scale production of monocillin I. Thus studies on optimization of its production by P. quadriseptata in different liquid media over varying time intervals were undertaken. The fungus was cultured in each of the four media, cultures neutralized to pH 7 with NaOH or HCl, extracted with EtOAc after 10, 15, and 20 days, and the resulting EtOAc extracts analyzed by HPLC for the content of monocillin I (1) as described in
"Materials and Methods" section. The results of the time course study in each of the four media, A-D are graphically presented in Fig. 1 and an HPLC trace typical of those used to quantitate the yield of monocillin I (1) is shown in Fig. 2. As apparent from Fig. 1, the highest yield (330mg/1000ml) of 1 was produced when P. quadriseptata was cultured in PDB prepared with tap water (medium B). Interestingly, production of secondary metabolites (as judged by the amount of EtOAc extract) as well as monocillin I by the organism was found to be inhibited in medium D. Further studies are in progress to identify minor non-cytotoxic metabolites (responsible for HPLC peaks 1~5 and 7~10; see Fig. 2) of P. quadriseptata.

In order to examine the contribution of various structural moieties for the cytotoxic activity of monocillin I (1), several of its derivatives were prepared and evaluated in all five cell lines. The structures of the derivatives 3~6 are based on their spectroscopic data. The regiospecific opening of the 7',8'-oxirane of monocillin I (1) during its hydrogenation to produce hexahydromonocillin I (6) with 8'-hydroxy group has literature precedence17) and was confirmed by the analysis of 1H-1H COSY and HSQC spectra of 6. The methyl doublet (J=6.6Hz) of 6 at δ 1.43 (assigned to H-11' on the basis of its chemical shift) showed 1H-1H correlation with the methine multiplet at δ 5.47 (assigned to H-10' based on 1H and 13C chemical shifts) which in turn was found to have 1H-1H correlations with the methyl protons at δ 1.98 (1H, ddd, J=14.8, 8.2, and 4.3 Hz, H-9'a) and 1.85 (1H, ddd, J=14.8, 7.7, and 2.9 Hz, H-9'b). Both these protons showed correlations to the proton at δ 3.72 (1H, m) in its 1H-1H COSY spectrum. In the HSQC spectrum it was found that this proton at δ 3.72 is on a methine carbon bearing an oxygen atom confirming that it is due to H-8'. The results of the cytotoxicity assay are summarized in Table 1. Both the monomethyl ether (3) and the dimethyl ether (4) of 1 retained activity although the latter was marginally more active than the former. The tetrahydro derivative 5 was moderately active against the cancer cell lines, but showed no cytotoxicity towards the normal cell line, WI-38. The hexahydro derivative 6 lacking the 7',8'-oxirane moiety was found to be completely devoid of any cytotoxicity suggesting the requirement of this functionality for the observed biological activity of monocillin I and its analogs.

Acknowledgments

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Table 1. Cytotoxicities of monocillin I (1) and its analogues 3~6 against a panel of tumor and normal cell lines.a

<table>
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<tr>
<th>Compound No.</th>
<th>NCI-H460</th>
<th>MCF-7</th>
<th>SF-268</th>
<th>MIA Pa Ca-2</th>
<th>WI-38</th>
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<td>6</td>
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<tr>
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</table>

aResults are expressed as IC50 values in μM. bKey: NCI-H460 = human non-small cell lung cancer; MCF-7 = human breast cancer; SF-268 = human CNS cancer (glioma); MIA Pa Ca-2 = human pancreatic cancer; WI-38 = human embryonic fibroblast cells. NA = Not active at 10 μg/ml.

References

curvularins of a *Penicillium* sp. from the rhizosphere of *Fallugia paradoxa* of the Sonoran desert. J. Antibiotics 57: 341–344, 2004


