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The Cytosporones, New Octaketide Antibiotics Isolated from an Endophytic Fungus

Sean F. Brady,[†] Melissa M. Wagenaar,[†] Maya P. Singh,[‡] Jeff E. Janso,[‡] and Jon Clardy*,[†]

Department of Chemistry and Chemical Biology, Cornell University, Ithaca, New York 14853, and Natural Products Microbiology, Wyeth-Ayerst Research, Pearl River, New York 10956

jcc12@cornell.edu

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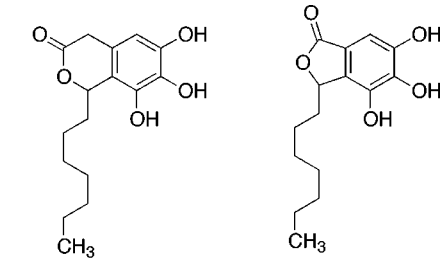
ABSTRACT



Organic extracts from cultures of endophytic fungi collected in the Guanacaste Conservation Area of Costa Rica were screened for antibiotic activity. Two endophytes CR200 (*Cytospora* sp.) and CR146 (*Diaporthe* sp.) were found to have potent antibiotic activity. Bioassay-guided fractionation of the extracts from these fungi led to the identification of cytosporones D and E, antibacterial active trihydroxybenzene lactones, and three related but inactive metabolites. The five new octaketides were characterized using X-ray crystallography and NMR.

Fungi are prodigious producers of biologically active natural products.[1] Since more than 1.5×10^6 endophytic fungi are now thought to live inside the estimated 270 000 species of vascular plants, the prospects for additional discoveries of interesting fungal metabolites are bright.[2,3]

In an ongoing effort to identify new biologically active secondary metabolites, we have characterized five new octaketides, trivially named the cytosporones, from the antibacterial active culture broth of two endophytic fungi, CR200 and CR146, from the Valsaceae family. CR200 (*Cytospora* sp.)[4] and CR146 (*Diaporthe* sp.)[4] were isolated from the tissue of *Conocarpus erecta* and *Forsteronia spicata* plants, respectively.[5] NMR and X-ray crystallography were used to characterize the structures of cytosporones D and E, two new cytotoxic trihydroxybenzene lactones, in addition to the inactive cytosporones A, B, and C.

Ethyl acetate extracts, which were active in antibacterial assays, of large-scale (10 L) CR200 fermentations grown in

[†] Cornell University.
[‡] Wyeth-Ayerst Research.

(1) (a) Pearce, C. *Adv. Appl. Microbiol.* **1997**, *44*, 1−80 and references therein. (b) Van Der Pyl, D.; Inokoshi, J.; Shiomi, K.; Yang, H.; Takeshima, H.; Omura, S. *J. Antibiot.* **1992**, *45*, 1802−1805. (c) Bergstrom, J. D.; Dufresne, C.; Bills, G.; Nallin-Omstead, M.; Byrne, K. *Annu. Rev. Microbiol.* **1995**, *49*, 607−639. (d) Singh, S. B.; Jones, E. T.; Goetz, M. A.; Bills, G. F.; Nallin-Omstead, M.; Jenkins, R. G.; Lingham, R. B.; Silverman, K. C.; Gibbs, J. B. *Tetrahedron Lett.* **1994**, *35*, 4693−4696.

(2) Dreyfuss, M. M.; Chapela, I. H. Potential of Fungi in the Discovery of Novel, Low-Molecular Weight Pharmaceuticals. In *The Discovery of Natural Products with Therapeutic Potential*; Gullo, V. P., Ed.; Butterworth−Heisman: Boston, 1994.

(3) Hawksworth, D. L. *Mycol. Res.* **1991**, *95*, 641−655.

(4) Both fungi were identified by comparing the rDNA internal transcribed spacer (ITS) regions amplified and sequenced from crude DNA extracts to sequences deposited in GenBank. The ITS region from CR200 is most closely related to sequences from *Cytospora* sp. while the ITS region from CR146 is most closely related to sequences from *Diaporthe* sp.

(5) The producing fungus was subcultured from the fungi that grew on potato dextrose agar from the interior of a small piece of branch that was surface sterilized by successive 5-min washes in 10% bleach, 70% EtOH, and sterile H_2O (3×).

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potato dextrose broth were subjected to a modified Kupchan scheme.[6] Cytosporones A, C, D, and E were partitioned from the CH_2Cl_2 fraction of the Kupchan scheme using three flash chromatography steps: C-18 (MeOH:H_2O) step gradient, silica gel (CH_2Cl_2:MeOH) step gradient, and C-18 (55:45 MeOH:H_2O) isocratic. Cytosporones A, C, D, and E were then purified from the active material that eluted from the final flash column using reversed phase C-18 HPLC (55:45 CH_3CN:H_2O + 0.1% acetic acid). Cytosporone B was purified from the CCl_4 fraction of the Kupchan scheme using two reversed phase chromatography steps: flash C-18 (CH_3-CN:H_2O) step gradient and reversed phase HPLC (55:45 CH_3CN:H_2O + 0.1% acetic acid). Although only cytosporones D and E show significant antibacterial activity, ^1H NMR, ^{13}C NMR, and HRMS data suggested that all five compounds were closely related.[7]

Cytosporone C crystallizes from acetone as colorless single crystals (0.40 × 0.40 × 0.05 mm^3) suitable for single-crystal X-ray diffraction analysis. The crystals belong to the centrosymmetric triclinic space group with lattice constants a = 4.843(6), b = 8.673(10), and c = 19.681(2) Å, α = 86.52(4), β = 89.91(2), and γ = 77.52(2)°, and Z = 2.[8] Cytosporone C is a dihydroxybenzene lactone with an *n*-heptane substituent (Figure 1) which crystallizes with its long hydrophobic tail in a fully extended conformation leading away from the bicyclic hydrophilic head. The space group in which it crystallizes requires cytosporone C to be isolated as a racemic mixture.[8]

Spectroscopic data are fully consistent with the crystal structure analysis of cytosporone C and were used to characterize the remaining cytosporones. ^{13}C chemical shift and $^1H-^{13}C$ DEPT NMR data confirm the presence of 1 methyl, 7 methylene, 1 oxygen-substituted methine, 6 aromatic, and 1 carbonyl carbon in cytosporone C. $^1H-^1H$ RelayH experiments defined the alkyl side chain, while $^1H-^{13}C$ HMBC correlations from the C-1 carbonyl and the C-3 and C-8 aromatic carbons to the H-9 methine and the H-2 methylene protons support the presence of the six-membered lactone. $^1H-^{13}C$ HMBC correlations from H-2, -4, and -9 to six independent aromatic carbon chemical shifts and long-range correlations from C-2, -5, and -6 to H-4 and from C-4, -5, -7, and -8 to H-6 complete the aromatic ring, confirming the entire cytosporone C carbon skeleton spectroscopically.

Similar 1- and 2-D NMR arguments can be used to show that the carbon skeleton of cytosporone D is identical to that of cytosporone C. However, the absence of one of the aromatic proton singlets in the ^1H spectrum and the additional oxygen observed by HRMS (HRMS−FAB (*m*/*z*): $[M + H]^+$ calcd for $C_{16}H_{23}O_5$ 295.1545; found 295.1544) suggest that cytosporone D contains a trihydroxy-substituted aromatic ring. Upon acetylation[9] three exchangeable protons (δ 9.22, 8.72, and 8.36 in d_6-DMSO) disappear and three new methyl singlets arise (δ 2.34, 2.28, 2.26 in d_6-DMSO), further confirming the presence of three hydroxyls in cytosporone D. The position of the remaining proton on the aromatic ring is defined as C-4 by long-range correlations from C-2 and -3 to H-4. Cytosporone D is therefore the 5,6,7-trihydroxybenzene derivative of cytosporone C.

Cytosporones D and E are equipotent antibiotics with similar ^1H and ^{13}C NMR spectra; however, HRMS (HRMS-EI (*m*/*z*): M^+ calcd for $C_{15}H_{20}O_5$ 280.1311; found 280.1308) indicates that cytosporone E is 14 mass units smaller than cytosporone D. $^1H-^{13}C$ DEPT and ^{13}C NMR experiments show that the C-2 methylene in cytosporone D is not present in cytosporone E. The lone aromatic proton in cytosporone

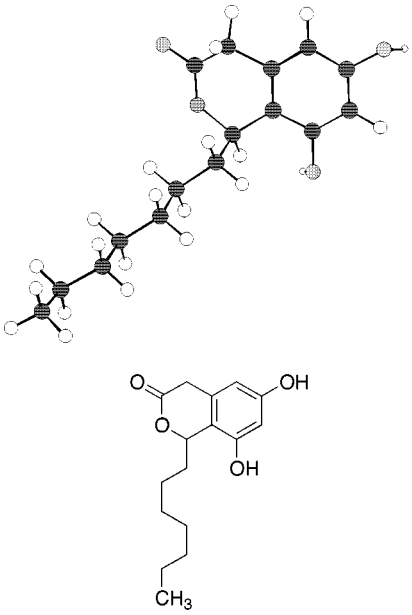
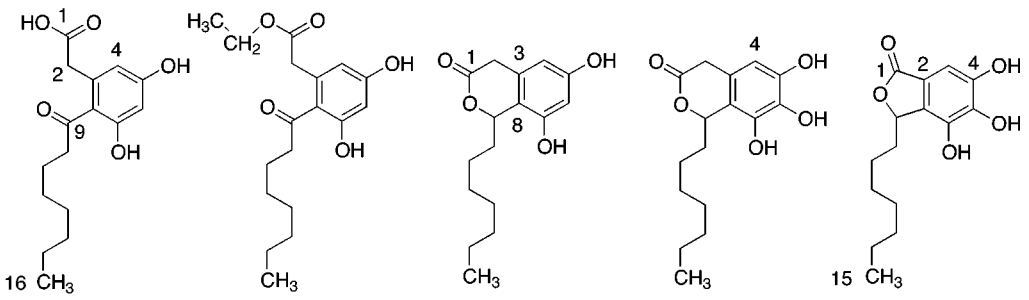


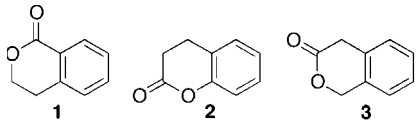
Figure 1. Computer-generated perspective drawing and chemical drawing of cytosporone C. Cytosporone C was isolated as a racemic mixture.



Cytosporone A Cytosporone B Cytosporone C Cytosporone D Cytosporone E

E is deshielded by 0.54 ppm relative to H-4 in cytosporone D, suggesting the presence of an ortho carbonyl substituted trihydroxybenzene moiety. The five-membered lactone in cytosporone E is suggested by the presence of an HMBC correlation from the C-1 carbonyl to H-3, which is not observed in the six-membered lactones cytosporones C and D. The five-membered lactone is further supported by a shift in the carbonyl IR stretch from 1716 cm^{-1} in cytosporone D to 1740 cm^{-1} in cytosporone E, which is characteristic of a five-membered lactone with an aryl group α to the carbonyl.

In place of the C-9 oxygen substituted methine present in cytosporone C, the ^{13}C NMR spectra of cytosporones A and B both show a C-9 ketone (δ 207.5 and 206.3, respectively) with HMBC correlations to the H-10 and -11 methylene protons. The additional C-9 oxidation suggests that the lactone present in cytosporone D is not present in cytosporones A or B. The C-1 carboxylic acid seen in cytosporone A is confirmed by the additional oxygen observed by HRFABMS (HRMS−FAB (*m*/*z*): $[M + H]^+$ calcd for $C_{16}H_{23}O_4$ 279.1596; found 279.1604). HRFABMS data for cytosporone B (HRMS−FAB (*m*/*z*): $[M + H]^+$ calcd for $C_{18}H_{27}O_5$ 323.1858; found 323.1859) suggest the presence of an additional two carbon unit, which is seen as a deshielded methylene triplet and methyl doublet coupled to the C-1 carbonyl indicating that cytosporone B is the ethyl ester of cytosporone A.



Dihydroisocoumarin (**1**)[10] and (dihydro)coumarin (**2**)[11] based compounds are commonly isolated as natural products. However, very few carbon skeletons based on a benzeneacetic acid derived lactone (**3**) have been reported to date.[12] Although structurally distinct from the curvularins, macrolactone dihydroxybenzenes,[12b] the cytosporones could be biosynthetically related to these common fungal metabolites. The curvularins are benzeneacetic acid based polyketide skeletons formally related to a cytosporone A-like precursor by the closure of the lactone at C-15 to give a 12-membered macrolactone instead of at C-9 to give the six-membered lactone seen in cytosporones C and D (Figure 2). These

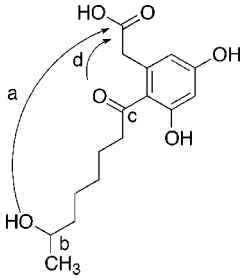


Figure 2. The cytosporone and curvularin skeletons are biosynthetically linked by a hypothetical cytosporone A-like intermediate. Curvularin requires the closure of the lactone at C-15 (a) while cytosporone requires reductions at C-15 (b) and C-9 (c) followed by closure of the lactone at C-9 (d).

compounds are all derived from a curvulinic acid type[12c,d] polyketide-folding pattern, which leads to a benzeneacetic

(6) The crude extract was resuspended at 1 mg/mL in 90:10 (MeOH:H_2O) and extracted three times with hexanes. The aqueous phase, following the addition of an additional 10% volume of H_2O, was re-extracted (3×) with CCl_4 and following the addition of another 10% volume of H_2O re-extracted (3×) with CH_2Cl_2.

(7) **Cytosporone A**: HRMS−FAB (*m*/*z*): $[M + H]^+$ calcd for $C_{16}H_{23}O_5$ 295.1545; found 295.1544; ^1H NMR acetone-d_6 6.36 (s, H4), 6.34 (s, H6), 3.64 (2H, s, H2), 2.94 (2H, t, 7.5, H10), 1.63 (p, H11), 1.27−1.30 (8H, bm, H12−H15), 0.86 (3H, t, 7, H16); ^{13}C NMR acetone-d_6 (C1−C16) 172.5, 40.6, 137.6, 102.5, 161.0, 111.7, 160.1, 120.5, 207.5, 44.4, 25.1, 30.0*, 29.9*, 32.5, 23.3, 14.3; UV λ_{max} (CH_3CN) 270, 219 nm; IR (NaCl, thin film) ν_{max} 3200, 1716, 1610, 1464, 1268, 1166, 1012 cm^{-1}. **Cytosporone B**: HRMS−FAB (*m*/*z*): $[M + H]^+$ calcd for $C_{18}H_{27}O_5$ 323.1858; found 323.1859; ^1H NMR acetone-d_6 6.37 (d, 2, H4), 6.31 (d, 2, H6), 4.08 (2H, q, 7, H17), 3.67 (2H, s, H2), 2.90 (2H, t, 7, H10), 1.63 (p, H11), 1.26−1.32 (8H, bm, H12−H15) 1.21 (3H, t, 7, H17), 0.88 (3H, t, 7, H16); ^{13}C NMR acetone-d_6 (C1−C18) 171.6, 40.1, 137.0, 102.5, 160.7, 111.8, 159.7, 120.8, 206.3, 44.4, 25.0, 30.0*, 29.9*, 32.5, 23.3, 14.3, 61.0, 14.5; UV λ_{max} (CH_3CN) 268, 219 nm; IR ($CHCl_3$) ν_{max} 3234, 1732, 1622, 1456, 1368, 1174 cm^{-1}. **Cytosporone C**: HRMS−FAB (*m*/*z*): $[M + H]^+$ calcd for $C_{16}H_{23}O_4$ 279.1596; found 279.1604; ^1H NMR acetone-d_6 6.34 (s, H4), 6.23 (s, H6), 5.55 (dd, 11.5, 3, H9), 3.79 (d, 24, H2), 3.45 (d, 24, H2), 1.86 (m, H10), 1.78 (m, H10), 1.55 (m, H11), 1.42 (m, H11), 1.27−1.30 (8H, m, H12−H15), 0.86 (3H, t, 8.5, H16); ^{13}C NMR acetone-d_6 (C1−C16) 170.5, 35.4, 133.5, 101.8, 159.0, 106.3, 154.5, 114.0, 78.3, 36.4, 26.4, 29.9, 29.9, 32.5, 23.3, 14.3; UV λ_{max} (CH_3CN) 278, 205 nm; IR ($CHCl_3$) ν_{max} 3298, 1726, 1622, 1468, 1356, 1146 cm^{-1}. **Cytosporone D**: HRMS−FAB (*m*/*z*): $[M + H]^+$ calcd for $C_{16}H_{23}O_5$ 295.1545; found 295.1544; ^1H NMR acetone-d_6 6.29 (s, H4), 5.57 (dd, 9, 6, H9), 3.71 (d, 19, H2), 3.39 (d, 19, H2), 1.86 (m, H10), 1.81, (m, H10), 1.55 (m, H11), 1.42 (m, H11), 1.26−1.33 (8H, bm, H12−H15), 0.88 (3H, t, 7, H16); ^{13}C NMR acetone-d_6 (C1−C16) 170.7, 34.8, 114.7, 106.2, 146.4, 132.2, 142.8, 122.1, 78.4, 36.5, 26.4, 29.9, 29.9, 32.5, 23.3, 14.3; UV λ_{max} (CH_3CN) 266, 205 nm; IR (NaCl, thin film) ν_{max} 3166, 1716, 1616, 1456, 1374, 1260, 1160, 1018 cm^{-1}. **Cytosporone E**: HRMS-EI (*m*/*z*): M^+ calcd for $C_{15}H_{20}O_5$ 280.1311; found 280.1308; ^1H NMR acetone-d_6 6.83 (s, H3), 5.46 (dd, 7, 3, H8), 2.24 (m, H9), 1.72 (m, H9), 1.23−1.44 (10H, m, H10−H14), 0.87 (3H, t, 7, H15); ^{13}C NMR acetone-d_6 (C1−C15) 170.9, 130.3, 102.8, 147.6, 139.5, 140.5, 118.1, 80.0, 33.8, 25.4, 30.6*, 29.9*, 32.5, 23.3, 14.3; UV λ_{max} (CH_3CN) 267, 217 nm; IR ($CHCl_3$) ν_{max} 3264, 1740, 1620, 1498, 1372, 1292, 1070 cm^{-1}. * indicates that assignments are interchangeable.

(8) A total of 1321 frames were taken on a Bruker SMART CCD Area detector diffractometer equipped with a 3 KW sealed tube (Mo Kα) X-ray generator using a narrow-frame method with scan widths of 0.3° in ω and an exposure time of 60 s/frame. Frames were integrated with the Bruker SAINT program to yield a total of 2714 reflections, of which 1638 were independent (R_{int} = 5.0%) and 1136 were above $4\sigma(F)$. Data were corrected for absorption using the SADABS program. The structure was solved by direct methods and refined by full matrix least squares on F^2 techniques using anisotropic displacement parameters for all non-hydrogen atoms. At final convergence, R_1 = 7.69% and GOF = 1.28 for 283 parameters. Crystallographic data for cytosporone C have been deposited with the Cambridge Crystallographic Data Center. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, U.K. (Fax: +44-(0) 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

(9) A total of 5 mg of cytosporone D was dissolved in 400 μL of 1:1 pyridine:acetic anhydride and stirred at 22 °C overnight. The acetylated product was then purified by reversed phase HPLC.

(10) McInerney, B. V.; Taylor, W. C. *Stud. Nat. Prod. Chem.* **1995**, *15*, 381−422.

(11) (a) Hoult, J. R. S.; Paya, M. *Gen. Pharmacol.* **1996**, *27*, 713−722. (b) Murray, R. D. H.; Mendez, J.; Brown, S. A. *The natural coumarins: occurrence, chemistry, and biochemistry*; Wiley: London, 1982.

(12) (a) Crawley, G. *J. Chem Soc., Perkin Trans. 1* **1981**, *59*, 221−223. (b) Birch, A. J.; Moore, B.; Rickards, R. W. *J. Chem. Soc.* **1962**, 220−222. (c) Kamal, A.; Qureshi, A. A.; Ahmad, A. *Tetrahedron* **1965**, *21*, 1411−1415. (d) Munro, H. D.; Musgrave, O. C.; Templeton, R. *J. Chem. Soc. C* **1967**, *10*, 947−948.

acid derived lactone. Cytosporone E is likely derived from the decarboxylation and subsequent oxidation of a cytosporone A-like precursor.

Cytosporones D and E show strong antibacterial activity while the closely related cytosporone C shows no significant antibacterial activity. The minimum inhibitory concentration for cytosporone D against representative strains of *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and the fungus *Candida albicans* is 8, 8, 64, and 4 μg/mL, respectively.[13] Against the same strains of bacteria and fungi, a gentamicin control showed minimum inhibitory concentrations of 2, 16, 2, and >128 μg/mL, respectively. The trihydroxybenzene, which appears in the antibacterial active cytosporones D and E but not cytosporone C, is likely to be the source of the antibiosis activity that these novel octaketide antibiotics exhibit.

(13) Cytosporone E was assayed against *S. aureus* where it was found to have the same activity as cytosporone D.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra for the cytosporones. Archival X-ray data for cytosporone C. This material is available free of charge via the Internet at http://pubs.acs.org.

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