Cytosporinols A–C, new caryophyllene sesquiterpenoids from Cytospora sp.

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Abstract: Three new caryophyllene sesquiterpenoids, cytosporinols A–C (1–3), have been isolated from solid cultures of Cytospora sp. The structures of 1–3 were elucidated primarily by NMR spectroscopy, and 3 was further confirmed by X-ray crystallography. The absolute configurations of the C-11 secondary alcohol in 1 and the 6,8-diol moiety in 3 were deduced using the modified Mosher and Snatzke’s method, respectively. Compounds 2 and 3 showed moderate cytotoxicity against HeLa cells.

Keywords: caryophyllene sesquiterpenoid, cytosporinol, Cytospora sp.

Introduction
Caryophyllene sesquiterpenoids have been isolated frequently from fungi as the bioactive principles. Examples include the pestalotiopsins, immunosuppressive and cytotoxic agents produced by the endophyte Pestalotiopsis sp. isolated from Taxus brevifolia;⁴,⁵ pestalotiopsolide A, teadolidol, and 6-epiteadolidol, highly oxidized caryophyllenes from a Pestalotiopsis sp. endophytic to the bark of Pinus taeda;⁶ the 6-hydroxypunctaporonins, antibacterial metabolites from a fungicolous Pestalotiopsis disseminata;⁷ fuscoatrol A, a cytotoxic agent from a marine-derived Humicola fuscoatra KMM 4629;⁸ the punctaporonins (also named as punctatins), antifungal agents from a coprophilous Poronia punctata;⁹ walleminol and wallleminone, two cis-fused iso-caryophyllenes from the toxigenic fungus Wallemia sebi;¹⁰ and Sch 725432, 601253, 601254, and 725434, antifungal agents from Chrysosporium pilosum.¹¹

During an ongoing search for new bioactive natural products from fungi of unique habitats, we initiated chemical studies of the fungi either inhabiting the fruiting body and larvae of Cordyceps sinensis⁴ or its surface soil.¹² As an extension, we also studied those species isolated from the soil samples that were collected on the Qinghai–Tibetan plateau at altitudes above 3,200 m, the environment in which Cordyceps sinensis was typically found. Our initial investigation of such an ascomyceteous fungus Cytospora sp. led to the isolation of three antimicrobial caryophyllene-derived meroterpenoids.¹³ Since the HPLC chromatogram of the crude extract revealed the presence of some minor components that could be the cytotoxic principles remained to be identified, the fungus was refermented in a larger scale using the same solid fermentation culture medium in which the meroterpenoids were first isolated.¹⁰ Fractionation of an EtOAc extract afforded three new caryophyllene sesquiterpenoids which we named cytosporinols A–C (1–3). Details of the isolation, structure elucidation, and cytotoxicity of these compounds are described herein.

Results and Discussion
Cytosporinol A (1) was assigned the molecular formula C₁₆H₂₁O₄ (five degrees of unsaturation) by HRESIMS. Analysis of its NMR data (Table 1) revealed two exchangeable protons (δH 3.37 and 3.93, respectively), four methyl groups (one O-methyl), two methylenes, six methines including four oxymethines, two sp³ quaternary carbons (one oxygenated), and one tetrasubstituted olefin. These data accounted for all the ¹H and ¹³C NMR resonances, suggesting that 1 was a tetracyclic compound. The ¹H–¹H COSY NMR data showed

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two isolated spin-systems of C-10–C-1–C-3 (including OH-11 and C-12) and C-6–C-7 (including OH-6). Interpretation of the HMBC data of 1 established a cyclobutane partial structure with two methyl groups attached to C-4. HMBC cross-peaks from H-6 to C-2 and from H-7 to C-5 indicated that C-6 is attached to the cyclobutane ring at C-5. The H-7 protons were correlated to C-8, C-9, and C-15, while the Me-15 protons were correlated to C-7, C-8, and C-9 in the HMBC spectrum of 1, linking the C-8 olefinic carbon (δC 134.5) to C-7, C-9 (δC 134.5), and C-15. HMBC correlations from H-10 to C-8 and C-12, H-11 to C-9, and from H-12 to C-8 connected C-9 to both C-10 and C-12, completing the partial structure with a cyclopentane moiety fused to a cyclopropane unit at C-1/C-9. The key HMBC correlation from H-12 to C-5 (δC 95.5) connected both C-5 and C-12 (δC 89.6) to the same oxygen atom to form a tetrahydrofuran (THF) ring fused to the cyclopentane and cyclopropane moieties at C-2/C-5 and C-1/C12, respectively. The HMBC correlation from the O-methyl proton signal Me-16 (δH 3.12) to the C-10 oxymethine carbon (δC 86.4) located the only methoxy group at C-10. On the basis of these data, the planar structure of 1 was established.

The relative configuration of 1 was deduced by analysis of the H–H coupling constants and NOESY data (Figure 1). The vicinal coupling constants observed for H-1 with H-11 and H-12 were 7.4 and 6.5 Hz, respectively, suggesting their cis-relative orientation,17 consistent with a similar THF moiety found in the known compound punctaporin C (4).18 NOESY correlations of Me-13 with H-2, H-3a, and H-6 indicated that these protons are all on the same face of the ring system, whereas those of H-3b with H-1 and Me-14, and of H-12 with Me-16 were used to place them on the opposite face. Collectively, these data allowed assignment of the relative configuration of 1. The 1H, 2R, 5S, 6S, 10R, 11S, and 12S absolute configurations were deduced for the stereogenic centers in 1 using the modified Mosher method19 as illustrated in Figure 2, which were also consistent with those assigned for corresponding stereogenic centers in 4.18

Cytosporinol B (2) was assigned the same molecular formula C20H20O4 as 1 by HRESIMS. Analysis of its NMR data (Table 2) revealed structural fragments similar to those presented in 1. However, the C-8–C-15 unit and the C-9 olefinic carbon (δC 134.5) were replaced by a terminal olefin (δH/δC 4.87/117.3, 144.7) and a methine (δH/δC 2.90/58.0), respectively, which were supported by relevant 1H–1H COSY and HMBC correlations. Therefore, the planar structure of 2 was proposed as shown. The relative configuration of 2 was also determined by analysis of the 1H–1H coupling constants and NOESY data (Figure 1). The coupling patterns and NOESY correlations for the relevant protons in 2 were nearly identical to those of 1, indicating that they possess the same relative configuration, except for the additional stereogenic center C-9, which was assigned by comparison of the coupling constant between H-9 and H-12 (9.7 Hz) with that reported for 4.18 This assignment was supported by NOESY correlations of H-9 with H-1 and Me-16. Considering the absolute configuration established for 1 by the modified Mosher method, C-9 was deduced to have the S configuration.

Cytosporinol C (3) gave a pseudomolecular ion [M + Na]+ peak by HRESIMS, consistent with a molecular formula C20H22O4 (four degrees of unsaturation). Analysis of its NMR data (Table 2) indicated that it is closely related to 1 and 2, except that the C-8/C-9 olefin in 1 or the C-8/C-15 terminal olefin in 2 was hydrated, and the OH-11 exchangeable proton (δH 3.93 in 1; 4.28 in 2) was replaced by a n-butyl group. These observations were supported by HMBC correlations from Me-15 to C-7, C-8, and C-9, and from the oxymethine proton H-11 to C-17, respectively. The relative configuration of 3 was assigned on the basis of NOESY data, and was confirmed by single crystal X-ray crystallographic analysis.
The absolute configuration of the cis-6,8-diol moiety in 3 was assigned using the in situ dimolybdenum CD method developed by Frelek. Upon addition of dimolybdenum tetaactate \([\text{Mo}_2\text{O}_7\text{Ac}_4]\) to a solution of 3 in DMSO, a metal complex was generated as an auxiliary chromophore. Due to the absence of other chromophores in 3, the observed sign of the Cotton effect in the induced spectrum originates solely from the chirality of the 6,8-diol moiety expressed by the sign of the “parallel” mode for Mo-O-C-C-O-Mo.22 When the molecule is viewed along the O-C bonds (Figure 4), the upper left and lower right sectors are defined as negative, whereas the upper right and lower left ones are defined as positive. The predicted sign of the Cotton effect depends upon the sign of the sector occupied by the largest part of the molecule. The negative Cotton effect observed at around 400 nm (band II) in the induced CD spectrum of 3 (Figure 5) permitted assignment of the 6S and 8R absolute configuration on the basis of the empirical rule proposed by Snatzke.20 Therefore, 3 was assigned 1R, 2R, 5S, 6S, 8R, 9S, 10R, 11R, and 12S absolute configuration.

\[\text{cis-6,8-diol moiety in 3} \]

\[\text{Figure 3. Thermal ellipsoid representation of 3} \]

Compounds 2 and 3 showed modest cytotoxicity against HeLa (cervical epithelium) cells, showing \(IC_{50}\) values of 16.5 and 21.1 \(\mu M\), respectively, while the positive control cisplatin showed an \(IC_{50}\) value of 7.6 \(\mu M\). However, compound 1 did not show noticeable cytotoxic effects against HeLa cells (\(IC_{50} > 100 \mu M\)).

Cytosporinols A–C (1–3) are closely related to the known taedolidol,\(^1\) 6-epitaedolidol,\(^1\) and punctaporin C (4),\(^3,6\) all possessing the rare oxatetracyclo[6.3.2.0\(^{5,12}\)]tridecane skeleton, but differ by having different substituents at C-6 (1), C-10 (1–3), and C-11 (3), as well as a tetrasubstituted olefin (1) and a terminal olefin (2). In addition, 1–3 are structurally similar to a synthetic byproduct 5 created in the acid-mediated

\[\text{Figure 4. Projection of the sector rule for the 6,8-diol moiety in 3} \]

\[\text{Table 2. NMR data for 2 (acetone-}\ \text{d}_2\ \text{and 3 (CDCl}_3\text{)} \]

| pos. | \(\delta^H (\text{in Hz})\) | HMBC\(^a\) | \(\delta^H (\text{in Hz})\) | HMBC\(^a\) | NOESY\(^a\) |
|------|-----------------|---------|-----------------|---------|----------|
| 1    | 2.74, dd (7.0, 5.8, 3.0) | 60.1, CH | 3, 9, 10 | 2.89, td (7.5, 3.3) | 56.8, CH | 3, 9, 10 | 3b  |
| 2    | 2.78, dd (9.4, 6.2, 5.0) | 34.8, CH | 3 | 2.46, td (9.8, 3.3) | 33.5, CH | 4, 6, 11 | 10 |
| 3a   | 1.87, dd (11.6, 9.4) | 41.2, CH\(_2\) | 1, 2, 4, 13 | 1.95, dd (11.3, 9.8) | 40.2, CH\(_2\) | 1, 2, 4, 5, 13, 14 |
| 3b   | 1.32, dd (11.6, 6.2) | 1, 2, 4, 5, 13, 14 | 1.36, dd (11.3, 9.8) | 1, 2, 4, 5, 13, 14 | 1, 14 |
| 4    | 37.5, qC | 96.0, qC | 37.3, qC |
| 5    | 3.93, d(t) (11.9, 3.2) | 68.4, CH | | 4.16, br | 71.4, CH | 8 | 15 |
| 6    | 2.65, dd (14.0, 3.2) | 38.6, CH\(_2\) | 8, 15 | 2.05, dd (16.0, 3.5) | 40.3, CH\(_2\) | 9 | 10 |
| 7a   | 2.19, dd (14.0, 3.2) | | 1.85, dd (16.0, 3.5) | 5, 6, 8, 9, 15 |
| 8    | | 144.7, qC | | 76.0, qC |
| 9    | 2.90, dd (9.7, 5.0) | 58.0, CH | 8, 10, 12 | 2.25, t (7.5) | 56.9, CH | 1, 7, 8, 10, 12, 15 |
| 10   | 3.58, t (5.0) | 92.1, CH | 8, 11, 12, 16 | 3.26, t (7.5) | 87.7, CH | 8, 9, 11, 16 | 2, 7a, 15, 17 |
| 11   | 4.04, d (5.8, 5.0, 4.3) | 76.3, CH | 2, 10 | 3.72, t (7.5) | 84.4, CH | 2, 10, 17 | 16 |
| 12   | 4.81, dd (9.7, 7.0) | 84.8, CH | 2, 5 | 4.98, t (7.5) | 83.9, CH | 2, 5, 8 |
| 13   | 1.17, s | 26.7, CH\(_2\) | 3, 4, 5, 14 | 1.21, s | 26.4, CH\(_3\) | 3, 4, 5, 14 |
| 14   | 0.97, s | 24.6, CH\(_2\) | 3, 4, 5, 13 | 1.08, s | 24.5, CH\(_3\) | 3, 4, 5, 13 | 3b |
| 15a  | 4.87, d (4.0) | 117.3, CH\(_2\) | 7, 9 | 1.19, s | 30.0, CH\(_3\) | 7, 8, 9 | 6, 10 |
| 15b  | 4.81, d (4.9) | | 7, 9 |
| 16   | 3.27, s | 57.5, CH\(_3\) | 10 | 3.35, s | 57.5, CH\(_3\) | 10 | 11 |
| 17   | | 3.34, d (6.3) | 69.4, CH\(_3\) | 11, 18, 19 | 10 |
| 18   | | 1.52, m | 32.0, CH\(_3\) | 17, 19, 20 | |
| 19   | | 1.35, m | 19.4, CH\(_3\) | 17, 18, 20 | |
| 20   | | 0.90, t (7.4) | 13.8, CH\(_3\) | 18, 19 | |
| OH-6 | 2.32, d (11.9) | | 3.37, d (4.8) | 5, 6 |
| OH-11| 4.28, d (4.3) | | | |

\(^a\)Recorded at 500 MHz; \(^b\)Recorded at 100 MHz; \(^c\)HMBC correlations, optimized for 8 Hz, are stated from proton(s) to the indicated carbons; \(^d\)Recorded at 400 MHz.
cyclization of the nonnatural enantiomer pestalotiopins,\(^ {23,24}\) but differ in having different configurations for all stereogenic centers and substituents at C-6 and C-11, respectively. Biogenetically, 1–3 could be derived from co-isolated fuscoatrol A\(^ {1}\) as proposed for the cytosporolides,\(^ {16}\) first via addition and cyclization to form 6, an intermediate of the C-6 deacetyl of (+)-pestalotiopsin A,\(^ {1}\) and then the acid-catalyzed cyclizations of 6 via two intermediates 7 and 8 could afford the key putative intermediate 9,\(^ {23}\) from which 1–3, taedolidol,\(^ {1}\) 6-epiptaedolidol,\(^ {1}\) and punctaporin C (4)\(^ {3,9}\) could be generated via different routes (Scheme 1).

**Figure 5.** CD spectrum of 3 in DMSO containing Mo\(_2\)(OAc)_4.

**Scheme 1.** Hypothetical biosynthetic pathways for compounds 1–3

**Experimental Section**

**General Experimental Procedures.** Optical rotations were measured on a Perkin-Elmer 241 polarimeter, and UV data were recorded on a Shimadzu Bipspec-1601 spectrophotometer. CD spectra were recorded on a JASCO J-815 spectropolarimeter. IR data were recorded using a Nicolet Magna-IR 750 spectrophotometer. \(^ {1}\)H and \(^ {13}\)C NMR data were acquired with Varian Mercury-400 and -500 spectrometers using solvent signals (acetone-\(_d_2\): \(\delta_H 2.05/\delta_C 29.8, 206.1;\) pyridine-\(_d_5\): \(\delta_H 7.21, 7.58, 8.73;\) CDCl\(_3\): \(\delta_H 7.26/\delta_C 76.7)\) as references. The HMQC and HMBC experiments were optimized for 145.0 and 8.0 Hz, respectively. ESIMS data were recorded on a Bruker Esquire 3000\textsuperscript{plus} spectrometer, and HRESIMS data were obtained using Bruker APEX III 7.0 T and APEX II FT-ICR spectrometers, respectively.

**Fungal Material.** The culture of *Cystospora* sp. was isolated by Yang Hao from a soil sample collected on the Qinghai-Tibetan plateau at an altitude above 3,200 m, Linzhi, Tibet, China, in April, 2007. The isolation and identification of the strain have been previously described.\(^ {19}\) The fungus was cultured on slants of PDA at 25 °C for 10 days. Agar plugs were cut into small pieces (about 0.5 × 0.5 × 0.5 cm\(^3\)) under aseptic conditions, 15 pieces were used to inoculate in three Erlenmeyer flasks (250 mL), each containing 50 mL of media (0.4% glucose, 1% malt extract, and 0.4% yeast extract); the final pH of the media was adjusted to 6.5 and sterilized by autoclave. Three flasks of the inoculated media were incubated at 25 °C on a rotary shaker at 170 rpm for five days to prepare the seed culture. Spore inoculum was prepared by suspending the seed culture in sterile, distilled H\(_2\)O to give a final spore/cell suspension of \(1 \times 10^9 /mL\) determined by microscope and hemocytometer. Fermentation was carried out in 12 Fernbach flasks (500 mL) each containing 80 g of rice. Distilled H\(_2\)O (120 mL) was added to each flask, and the contents were soaked overnight before autoclaving at 15 psi for 30 min. After cooling to room temperature, each flask was inoculated with 5.0 mL of the spore inoculum and incubated at 25 °C for 40 days.

**Extraction and Isolation.** The fermented material was extracted with EtOAc (4 × 1.0 L), and the organic solvent was evaporated to dryness under vacuum to afford a crude extract (6.0 g). The extract was fractionated by silica gel VLC using petroleum ether–EtOAc gradient elution. The fraction (150 mg) eluted with 50% EtOAc was separated by Sephadex LH-20 column chromatography (CC) eluting with 1:1 CH\(_2\)Cl\(_2\)–MeOH. The resulting subfractions were combined and further purified by semipreparative RP HPLC (Agilent Zorbax SB-C\(_{18}\) column; \(5 \mu m; 9.4 \times 250 \text{mm; } 2 \text{ mL/min}\)) to afford cytosporins A (1; 6.4 mg; \(t_R 19.93 \text{ min; } 45\% \text{CH}_2\text{CN in H}_2\text{O for 25 min})\) and B (2; 2.0 mg, \(t_R 17.00 \text{ min; } 25\% \text{CH}_2\text{CN in H}_2\text{O for 20 min}\)). The fraction (300 mg) eluted with 30% EtOAc was eluted again through Sephadex LH-20 CC (1:1 CH\(_2\)Cl\(_2\)–MeOH) to afford cytosporinol C (3; 60.0 mg).

**Cytosporolin A (1):** colorless oil; \([\alpha]_D^{23} +86 \text{ (c 0.1, MeOH); UV (MeOH)} \lambda_{max} (\log \epsilon) 208 (3.36) \text{ nm; IR ( neat) } \nu_{max} 3424 \text{ (br), } 2933, 2866, 1709, 1446, 1320, 1094, 1064, 1054, 1011, 975, 949 \text{ cm}^{-1}; \text{ H, } ^{13}\text{C NMR, and HMBC data see Table 1; positive ion ESIMS } m/z 303 \text{ [M + Na]}^+; \text{ HRESIMS } m/z 303.1564 \text{ (calcd for C}_{19}\text{H}_{23}\text{O}_5\text{Na, 303.1567).**}

**Preparation of (R)-(1a) and (S)-MTPA (1b) Esters.**\(^ {19}\) A sample of 1 (2.0 mg, 0.007 mmol), (S)-MTPA CI (3.0 \mu L, 0.017 mmol), and pyridine-\(_d_5\) (0.5 mL) were allowed to react in an NMR tube at ambient temperature for 24 h, with the \(^1\)H NMR data of the R-MTPA ester derivative (1a) were obtained directly on the reaction mixture: \(^1\)H NMR (pyridine-\(_d_5\), 400 MHz) \(\delta 5.54 \text{ (1H, d, } J = 4.0 \text{ Hz, H-12), } 5.51 \text{ (1H, d, } J = 8.0 \text{ Hz, H-11), } 4.32 \text{ (1H, s, H-10), } 4.17 \text{ (1H, dd, } J = 12, \text{ 4.0 Hz, H-6), } 3.70 \text{ (1H, t, } J = 12 \text{ Hz, H-7a), } 3.23 \text{ (3H, s, Me-16), } 2.90 \text{ (1H, td, } J = 8.0, 2.5 \text{ Hz, H-2), } 2.62 \text{ (1H, ddd, } J = 8.0, 4.0, 2.5 \text{ Hz, H-1), } 2.16 \text{ (1H, dd, } J = 12, \text{ 4.0 Hz, H-7b), } 1.91 \text{ (1H, dd, } J = 12, 8.0 \text{ Hz, H-3a), } 1.74 \text{ (3H, s, Me-15), } 1.57 \text{ (3H, s, Me-13), } 1.54 \text{ (1H, dd, } J = 12, 8.0 \text{ Hz, H-3b), } 1.32 \text{ (3H, s, Me-14).**}
Similarly, the reaction mixture from another sample of 1 (2.0 mg, 0.007 mmol), (R)-MTPA CI (3.0 µL, 0.017 mmol), and pyridine-d5 (0.5 mL) was processed as described above for 1a to afford 1b: 1H NMR (pyridine-d5, 400 MHz) δ 5.56 (1H, d, J = 4.0 Hz, H-12), 5.55 (1H, d, J = 8.0 Hz, H-11), 4.26 (1H, dd, J = 12, 4.0 Hz, H-6), 4.19 (1H, s, H-10), 3.70 (1H, t, J = 8.0, 2.5 Hz, H-2), 2.91 (1H, dd, J = 8.0, 4.0, 2.5 Hz, H-11), 2.15 (1H, dd, J = 12, 4.0 Hz, H-7b), 2.00 (1H, m, H-3b), 1.65 (3H, s, Me-15), 1.61 (3H, s, Me-13), 1.35 (3H, s, Me-14).

Cytosporinol B (2): colorless oil; [α]24° 2.8 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 208 (3.38) nm; IR (neat) νmax 3423 (br), 2936, 1718, 1445, 1382, 1236, 1104, 1043, 968 cm⁻¹; 1H, 13C NMR, and HMBC data see Table 2; positive ion ESIMS m/z 303 [M + Na⁺]; HRESIMS m/z 315.1656 (calf for C13H14O5Na, 303.1567).

Cytosporinol C (3): colorless needles (MeOH–H2O, mp 149–152 °C; [α]20° 8.8 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 209 (3.38) nm; IR (neat) νmax 3473, 3444, 2960, 2930, 2874, 1713, 1466, 1401, 1385, 1235, 1150, 1133, 1118, 1093, 1067, 1038, 1000, 901 cm⁻¹; 1H, 13C NMR, and NOESY data see Table 2; positive ion ESIMS m/z 377 [M + Na⁺]; HRESIMS m/z 377.2300 (calf for C16H16O6Na, 377.2298).

X-ray Crystallographic Analysis of 3. Upon crystallization from MeOH–H2O (10:1) using the vapor diffusion method, colorless crystals were obtained for 3. A crystal (0.40 × 0.30 × 0.08 mm) was separated from the sample and mounted on a glass fiber, and data were collected using a Rigaku Saturn CCD area detector with graphite-monochromated Mo Kα radiation, λ = 0.71073 Å at 173(2) K. Crystal data: C25H20O16, M = 534.47, space group orthorombic, P212121; unit cell dimensions a = 5.8938 (17) Å, b = 13.939 (4) Å, c = 23.127 (7) Å, V = 1900.0 (10) Å³; Z = 4, Dcalc = 1.239 mg·cm⁻³, µ = 0.087 mm⁻¹, F(000) = 776. The structure was solved by direct methods using SHELXL-97 and refined using full-matrix least-squares difference Fourier techniques. All non-hydrogen atoms were refined with anisotropic displacement parameters, and all hydrogen atoms were placed in idealized positions and refined as riding atoms with the relative isotropic parameters. Absorption corrections were applied with the Siemens Area Detector Absorption Program (SADABS). The 12519 measurements yielded 2026 independent reflections after equivalent data were averaged, and Lorentz and polarization corrections were applied. The final refinement gave R1 = 0.0495 and wR2 = 0.0990 [I > 2σ(I)].

Absolute Configuration of the 6,8-Diol Moiety in 3. HPLC grade DMSO was dried with 4 Å molecular sieves. According to a published procedure, a mixture of 1:2 diol/Mo(OAc)4 for 3 was subjected to CD measurements at a concentration of 0.6 mg/mL. The first CD spectrum was recorded immediately after mixing, and its time evolution was monitored until stationary (about 10 min after mixing). The inherent CD was subtracted. The observed sign of the diagnostic band at around 400 nm in the induced CD spectrum was correlated to the absolute configuration of the 6,8-diol moiety.

MTS Assay. The assay was run in triplicate. In a 96-well plate, each well was plated with 2–5 × 10³ cells (depending on the cell multiplication rate). After cell attachment overnight, the medium was removed, and each well was treated with 50 µL medium containing 0.1% DMSO, or appropriate concentrations of the test compounds and the positive control cisplatin (100 mM as stock solution of a compound in DMSO and serial dilutions; the test compounds showed good solubility in DMSO and did not precipitate when added to the cells). The plate was incubated for 72 h at 37 °C in a humidified, 5% CO₂ atmosphere. Proliferation assessed by adding 20 µL of MTS (Promega) to each well in the dark, followed by a 90 min incubation at 37 °C. The assay plate was read at 490 nm using a microplate reader.

Electronic Supplementary Material
Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s13659-012-0018-z and is accessible for authorized users.

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