Research Article

Punctaporonins N–S, New Caryophyllene Sesquiterpenoids from Cytospora sp.

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Received 5 May 2017; Revised 27 July 2017; Accepted 22 August 2017; Published 24 October 2017

Academic Editor: Jane Hanrahan

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Six new caryophyllene sesquiterpenoids, punctaporonins N–S (1–6), and three known ones, 6-hydroxypunctaporonins B (7), A (8), and E (9), have been isolated from solid cultures of Cytospora sp. The structures of 1–6 were elucidated primarily by NMR spectroscopy. The absolute configuration of 1 was assigned by X-ray crystallographic analysis of its S-MTPA ester. Compounds 2, 5, and 6 showed modest cytotoxicity against HeLa cells.

1. Introduction

Fungi are capable of producing a variety of bioactive secondary metabolites [1]. Since the secondary metabolism of fungi may be influenced by selection pressures exerted by other organisms and the environment in which they reside, those species thriving in unique and competitive niches are especially likely to produce bioactive natural products with diverse and interesting structural features [2, 3]. Based on this consideration and the documented success in finding new bioactive natural products from special types of fungi [4], we initiated chemical studies of the fungi inhabiting either the fruiting body and larvae of Cordyceps sinensis [5–10] or its surface soil [11–13]. 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. During the course of our continuing search for new bioactive natural products from this kind of fungal species, an ascomycetous fungus Cytospora sp. was isolated from a soil sample that was collected at Linzhi, Tibet, China. Our initial investigation of this fungus that was fermented in rice medium at 25°C led to the isolation of three antimicrobial caryophyllene-derived meroterpenoids [14, 15] and three cytotoxic caryophyllene sesquiterpenoids [16]. In the current work, the fungus was fermented in the same solid culture medium at 15°C, and the HPLC chromatogram of the crude extract revealed the presence of additional components. Fractionation of an EtOAc extract afforded six new caryophyllene sesquiterpenoids, which we named punctaporonins N–S (1–6), and three known ones, 6-hydroxypunctaporonins B (7), A (8), and E (9) (Figure 1) [17]. Details of the isolation, structure elucidation, and cytotoxicity of these compounds are described herein.

2. Materials and Methods

2.1. General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, and UV data were recorded on a Shimadzu Biospec-1601 spectrophotometer. IR data were recorded using a Nicolet Magna-IR 750 spectrophotometer. 1H and 13C NMR data were acquired with Varian Mercury-400, Varian Mercury-500, and Varian Mercury-600 MHz spectrometers using solvent signals (acetone-d6: δH 2.05/δC 29.8, 206.1; pyridine-d5: δH 7.21, 7.58, 8.73) as references. The HMQC and HMBC experiments were optimized for 145.0 and 8.0 Hz, respectively. ESIMS data and
HRESIMS data were obtained using an Agilent Accurate-Mass-Q-TOF LC/MS 6520 instrument equipped with an electrospray ionization (ESI) source. The fragmentor and capillary voltages were kept at 125 and 3500 V, respectively. Nitrogen was supplied as the nebulizing and drying gas (300°C). The flow rate of the drying gas and the pressure of the nebulizer were 10 L/min and 10 psi, respectively. All MS experiments were performed in positive ion mode. Full-scan spectra were acquired over a scan range of m/z 100–1000 at 1.03 spectra/s. All solvents used were of analytical grade. Column chromatography was performed with silica gel (100–200 or 200–300 mesh, Qingdao Marine Chemical Inc., China) and Sephadex LH-20 (GE, USA). Semipreparative HPLC was performed on an Agilent 1260 G1365D MWD detector and an Agilent Zorbax SB-C18 column (5 µm; 9.40 mm × 250 mm).

2.2. Fungal Material and Fermentation. The isolation, identification, and fermentation of the fungus *Cytospora* sp. were the same as those we previously described [14–16], except the incubation temperature was changed to 15°C.

2.3. 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 (4.2 g). The extract was fractionated by silica gel VLC using petroleum ether–EtOAc gradient elution. The fraction (100 mg) eluted with 35% EtOAc was separated by Sephadex LH-20 column chromatography (CC) eluting with 1:1 CH2Cl2–MeOH. The resulting subfractions were combined and further purified by semipreparative RP HPLC (59% MeOH in H2O for 35 min; 2 mL/min) to afford punctaporonins N (1: 4.5 mg, tR 21.22 min), O (2: 3.5 mg, tR 31.33 min), P (3: 2.0 mg, tR 14.05 min), and R (5: 0.8 mg, tR 24.22 min). The fraction (50 mg) eluted from the silica gel column with 45% EtOAc was purified by RP HPLC (35% CH3CN in H2O for 20 min; 2 mL/min) to afford punctaporonin S (6: 2.0 mg; tR 17.02 min). The fraction (150 mg) eluted from the silica gel column with 50% EtOAc was fractionated again by Sephadex LH-20 CC eluting with 1:1 CH3Cl2–MeOH. Purification of the resulting subfractions with different gradients afforded 6-hydroxypunctaporonins B (7; 10.0 mg, tR 23.61 min; 50% MeOH in H2O for 30 min), A (8: 8.4 mg, tR 16.12 min; same gradient as in purification of 7), and E (9: 3.5 mg, tR 14.83 min; same gradient as in purification of 7).

2.4. Identification

**Punctaporonin N (1)**. White powder (MeOH); [α]D 26 = −73 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 198 (3.40) nm; IR (neat) νmax 3317 (br), 2936, 2870, 1736, 1714, 1463, 1372, 1241, 1095, 1037 cm−1; 1H, 13C NMR, HMBC, and NOESY data see Table 1; (+)–HR–ESI–MS m/z 305.1719 [M + Na]+ (calcd. for C16H16O5Na, 305.1723).

**Punctaporonin O (2)**. Colorless oil (MeOH); [α]D 26 = −47 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 202 (3.58) nm; IR (neat) νmax 3263 (br), 2932, 2875, 1713, 1463, 1361, 1181, 1089, 1058, 1029 cm−1; 1H and 13C NMR data see Table 2; (+)–HR–ESI–MS m/z 305.1721 [M + Na]+ (calcd. for C16H16O5Na, 305.1723).

**Punctaporonin P (3)**. Colorless oil (MeOH); [α]D 26 = −71 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 203 (3.55) nm; IR (neat) νmax 3262 (br), 2958, 2935, 2872, 1735, 1717, 1372, 1240, 1089, 1021 cm−1; 1H and 13C NMR data see Table 2; (+)–HR–ESI–MS m/z 333.1669 [M + Na]+ (calcd. for C17H18O4Na, 333.1672).

**Punctaporonin Q (4)**. Colorless oil (MeOH); [α]D 26 = −37 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 203 (3.60) nm; IR (neat) νmax 3438 (br), 2957, 2936, 1730, 1715, 1381, 1249, 1031 cm−1; 1H and 13C NMR data see Table 2; (+)–HR–ESI–MS m/z 333.1676 [M + Na]+ (calcd. for C17H18O4Na, 333.1672).

**Punctaporonin R (5)**. Colorless oil (MeOH); [α]D 26 = −30 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 200 (3.22) nm; IR (neat) νmax 3256 (br), 2957, 2934, 2869, 1711, 1462, 1365, 1102, 1057, 1031, 984 cm−1; 1H and 13C NMR data see Table 2; (+)–HR–ESI–MS m/z 333.1672 [M + Na]+ (calcd. for C17H18O4Na, 333.1672).

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**Figure I**: Structures of compounds 1–9.
Table 1: NMR data (400 MHz, Acetone-\(d_6\)) for punctaporin N (1).

| Position | \(\delta_C\), mult. | \(\delta_H\) (J in Hz) | HMBC* | NOESY |
|----------|------------------|----------------------|--------|--------|
| 1        | 140.0, qC        |                      |        |        |
| 2        | 39.8, CH         | 3.39, dd (11.9, 8.2) | 1, 3, 5, 11, 12 | 13     |
| 3a       | 34.0, CH_2       | 2.15, dd (11.9, 9.8) | 1, 2, 4, 13, 14 |        |
| 3b       |                  | 1.49, dd (9.8, 8.2)  | 2, 4, 5, 13 | 13     |
| 4        | 41.0, qC         |                      |        |        |
| 5        | 82.7, qC         |                      |        |        |
| 6        | 71.4, CH         | 3.86, br d (4.7)    |        |        |
| 7a       | 42.5, CH_2       | 2.63, dd (16.3, 4.7) | 5, 6, 15 |        |
| 7b       |                  | 1.64, dt (16.3, 2.0) | 6, 8, 9, 15 |        |
| 8        | 78.4, qC         |                      |        |        |
| 9        | 141.9, CH        | 5.70, dt (13.0, 2.0) | 7, 10, 11 |        |
| 10       | 124.5, CH        | 5.62, d (13.0)      | 1, 9   |        |
| 11       | 125.9, CH        | 5.88, s             | 1, 2, 9, 12 | 12b   |
| 12a      | 64.8, CH_2       | 4.19, d (12.0)      | 1, 11  |        |
| 12b      |                  | 3.93, d (12.0)      | 1, 2, 11 | 11, 13 |
| 13       | 24.6, CH_2       | 1.23, s             | 3, 4, 5, 14 | 2, 3b, 6, 12b |
| 14       | 24.1, CH_2       | 1.07, s             | 3, 4, 5, 13 | OH-5  |
| 15       | 26.0, CH_2       | 1.10, s             | 7, 8, 9 | 16     |
| 16       | 48.7, CH_3       | 3.26, s             | 8      | 6, 15  |
| OH-5     |                  | 4.53, br s          |        | 14     |
| OH-6     |                  | 4.67, br s          |        |        |
| OH-12    |                  | 3.14, br s          |        |        |

*HMBC correlations, optimized for 8 Hz, are from proton(s) stated to the indicated carbon.

Table 3: (+)–HR–ESI–MS m/z 305.1717 [M + Na]+ (calcd. for C_{16}H_{26}O_{4}Na, 305.1723).

Punctaporin S (6). Colorless oil (MeOH); [\(\alpha\)]_{D}^{25} = –84 (c 0.1, MeOH); UV (MeOH) \(\lambda_{max} (\log \varepsilon)\) 202 (3.56) nm; IR (neat) \(\gamma_{max}\) 3486 (br), 2953, 2938, 2871, 1736, 1574, 1252, 1040, 990 cm\(^{-1}\); \(1^H\) and \(13^C\) NMR data see Table 3; (+)–HR–ESI–MS m/z 375.1781 [M + Na]+ (calcd. for C_{19}H_{28}O_{6}Na, 375.1778).

6-Hydroxy-punctaporin B (7). \(1^H\), \(13^C\) NMR, and the MS data were consistent with literature values [17].

6-Hydroxy-punctaporin A (8). \(1^H\), \(13^C\) NMR, and the MS data were consistent with literature values [17].

6-Hydroxy-punctaporin E (9). \(1^H\), \(13^C\) NMR, and the MS data were consistent with literature values [17].

2.5. Preparation of (S)-MTPA Ester (1a). A sample of 1 (2.0 mg, 0.007 mmol) was dissolved in pyridine (2.0 mL) in a 10 mL round-bottomed flask. (R)-MTPA Cl (2.0 \(\mu\)L, 0.011 mmol) were quickly added, and the flask was sealed and all contents were stirred in room temperature for 12 h. The mixture was evaporated to dryness and purified by semi-preparative HPLC (90% CH_{3}OH in H_{2}O for 20 min; 2 mL/min) to afford 1a (2.2 mg, \(t_{R}\) 15.72 min); colorless platelets; \(1^H\) NMR (pyridine-\(d_5\); 500 MHz) \(\delta\) 6.16 (IH, s, H-11), 6.00 (IH, d, \(J = 13\) Hz, H-9), 5.75 (IH, d, \(J = 13\) Hz, H-10), 5.55 (IH, d, \(J = 12\) Hz, H-12a), 5.46 (IH, d, \(J = 12\) Hz, H-12b), 4.37 (IH, s, H-6), 3.35 (3H, s, H-16), 3.09 (IH, d, \(J = 10\) Hz, H-2), 2.34 (IH, t, \(J = 10\) Hz, H-3a), 2.11 (IH, d, \(J = 16\) Hz, H-7a), 1.54 (IH, t, \(J = 10\) Hz, H-3b), 1.35 (IH, d, \(J = 16\) Hz, H-7b), 1.37 (3H, s, H-13), 1.37 (3H, s, H-14), 1.18 (3H, s, H-3s), 1.15 (3H, s, H-3s).

2.6. X-Ray Crystallographic Analysis of 1a [18]. Upon crystallization from MeOH/H_{2}O (10:1) using the vapor diffusion method, colorless crystals were obtained for 1a, and a crystal (0.90 \(\times\) 0.27 \(\times\) 0.08 mm\(^3\)) 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 K\(\alpha\) radiation, \(\lambda = 0.71073\) \(\AA\) at 173(2) K. Crystal data: C_{26}H_{33}F_{3}O_{6}\_H_{2}O, \(M = 516.54\), space group orthorhombic, P2\(\text{I}_{1}\)2\(\text{I}_{1}\); unit cell dimensions \(a = 6.9639\) (14) \(\text{Å}, b = 17.211\) (3) \(\text{Å}, c = 21.948\) (5) \(\text{Å}, V = 2630.5\) (9) \(\text{Å}^3\), \(Z = 4\), \(D_{calc} = 1.304\) mg\(^\text{\text{\text{\text{\text{m}}}}}^3\), \(\mu = 0.107\) mm\(^{-1}\), \(F(000) = 1096\). The structure was solved by direct methods using SHELXL-2016 [19] and refined using full-matrix least-squares difference Fourier techniques. All nonhydrogen 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) [20]. The 14274 measurements yielded 4799 independent reflections after equivalent data were averaged, and Lorentz and polarization corrections were applied. The final refinement gave \(R_{1} = 0.0811\) and \(wR_{2} = 0.1232\) [\(I > 2\sigma(I)\)].
3. Results and Discussion

Punctaporin N (I) was obtained as a white powder with a molecular formula of C_{16}H_{26}O_{4} (four degrees of unsaturation), established by HRESIMS. Analysis of its NMR spectroscopic data (Table 1) revealed structural similarity to the coisolated known compound 6-hydroxypunctaporin O. The coupling constant of 13.0 Hz between H-9 and H-10, which was exactly the same as in 7, suggested the Z-geometry for the C-9/C-10 olefin [17]. The C-1/C-11 olefin was deduced to be E-configuration based on NOESY correlation of H-11 with H-12b. NOESY correlations of H_{3}-13 with H-2, H-3b, and H-6 and H-6 with H_{3}-16 indicated that these protons are all on the same face of the ring system, whereas that of H_{3}-14 with OH-5 was used to place them on the opposite face, thereby establishing the relative configuration of I.

The modified Mosher method was tried to assign the C-6 absolute configuration of I. However, treatment of I with (R) or (S)-MTPA Cl to form the C-6-MTPA ester was unsuccessful; instead the reaction products C-12 S-MTPA ester (1a) and C-12 R- MTPA ester (1b) were generated. In order to establish the absolute configuration of I, one of the reaction products C-12 S-MTPA ester (1a) was purified by HPLC to obtain a single crystal, and its perspective ORTEP plot is shown in Figure 3. Although the X-ray diffraction was collected with Mo Kα radiation, which only gave the relative configuration for 1a, the absolute configuration of the chiral center at C-18 generated from (R)-MTPA Cl had been assigned to be S. Therefore, the absolute configuration of 1 was established as 2R, 5S, 6S, and 8S by single-crystal X-ray crystallographic analysis of its C-12 S-MTPA ester (1a).

Punctaporin O (2) was assigned the same molecular formula C_{16}H_{26}O_{4} as I by HRESIMS. Its NMR data (Table 2) were recorded at 100 MHz. The coupling constant for the C-9/C-10 olefin [17] was exactly the same as in 7, suggested the Z-geometry for the C-9/C-10 olefin [17]. The C-1/C-11 olefin was deduced to be E-configuration based on NOESY correlation of H-11 with H-12b. NOESY correlations of H_{3}-13 with H-2, H-3b, and H-6 and H-6 with H_{3}-16 indicated that these protons are all on the same face of the ring system, whereas that of H_{3}-14 with OH-5 was used to place them on the opposite face, thereby establishing the relative configuration of I.

2.7. MTS Assay. The MTS assay method was the same as that we previously described [16, 21, 22].

### Table 2: NMR spectroscopic data for punctaporins O–Q (2–4) in acetone-d_6.

| Position | δ_C \(^{a}\), mult. | δ_H \(^{b}\) (J in Hz) | δ_C \(^{a}\), mult. | δ_H \(^{b}\) (J in Hz) | δ_C \(^{a}\), mult. | δ_H \(^{b}\) (J in Hz) |
|----------|-----------------|-------------------|-----------------|-------------------|-----------------|-------------------|
| 1        | 55.3, qC        | 55.6, qC          | 53.6, qC        | 53.6, qC          | 55.3, qC        | 55.6, qC          |
| 2        | 471.1, CH       | 2.54, dd (13.0, 8.0) | 472.2, CH       | 2.32, dd (12.8, 7.8) | 45.6, CH       | 2.35, dd (12.5, 7.9) |
| 3a       | 35.5, CH_2      | 2.02, dd (13.0, 8.0) | 35.5, CH_2      | 2.08, dd (12.8, 8.9) | 36.3, CH_2      | 2.13, dd (12.5, 9.2) |
| 3b       | 1.46, t (8.0)   | 1.52, dd (8.9, 7.8) | 1.46, dd (9.2, 7.9) | 1.46, dd (9.2, 7.9) | 1.46, dd (9.2, 7.9) | 1.46, dd (9.2, 7.9) |
| 4        | 42.8, qC        | 42.8, qC          | 42.7, qC        | 42.7, qC          | 42.7, qC        | 42.7, qC          |
| 5        | 80.6, qC        | 80.4, qC          | 81.7, qC        | 81.7, qC          | 81.7, qC        | 81.7, qC          |
| 6        | 68.9, CH        | 4.09, dd (10.0, 5.6) | 68.7, CH        | 4.06, ddd (10.3, 7.9, 5.6) | 68.9, CH        | 4.17, dd (10.0, 5.9) |
| 7a       | 41.5, CH_2      | 1.86, dd (13.5, 5.6) | 41.2, CH_2      | 1.77, dd (14.0, 5.6) | 40.3, CH_2      | 1.90, dd (13.5, 5.9) |
| 7b       | 1.56, dd (13.5, 10.0) | 1.62, dd (14.0, 10.3) | 1.64, dd (13.5, 10.0) | 1.64, dd (13.5, 10.0) | 1.64, dd (13.5, 10.0) | 1.64, dd (13.5, 10.0) |
| 8        | 51.0, qC        | 50.0, qC          | 50.9, qC        | 50.9, qC          | 50.9, qC        | 50.9, qC          |
| 9        | 973.3, CH       | 3.60, d (2.0)     | 89.4, CH        | 5.11, d (2.5)     | 86.5, CH        | 4.07, d (2.5)     |
| 10       | 1279, CH        | 5.90, dd (6.0, 2.0) | 126.6, CH       | 5.67, dd (5.9, 2.5) | 130.6, CH       | 5.68, dd (6.0, 2.5) |
| 11       | 143.4, CH       | 5.74, d (6.0)     | 145.3, CH       | 5.88, d (5.9)     | 141.2, CH       | 5.92, d (6.0)     |
| 12a      | 62.2, CH_2      | 3.68, d (11.4)    | 62.0, CH_2      | 3.73, d (11.2)    | 65.6, CH_2      | 4.57, d (11.6)    |
| 12b      | 3.40, d (11.4)  | 3.48, dd (11.2, 8.0) | 3.47, d (11.6)  | 3.47, d (11.6)    | 3.47, d (11.6)  | 3.47, d (11.6)    |
| 13       | 23.4, CH_3      | 1.11, s           | 23.4, CH_3      | 1.19, s           | 23.3, CH_3      | 1.10, s           |
| 14       | 24.1, CH_3      | 1.08, s           | 24.0, CH_3      | 1.12, s           | 23.7, CH_3      | 1.05, s           |
| 15       | 24.6, CH_3      | 1.05, s           | 24.3, CH_3      | 1.10, s           | 26.2, CH_3      | 0.84, s           |
| 16       | 57.5, CH_3      | 3.33, s           | 4.82, br s      | 4.88, s           | 4.59, br s      | 4.37, d (7.9)     |
| OH-5     | 5.39, s         | 5.37, d (7.9)     | 5.37, d (7.9)   | 5.37, d (7.9)     | 5.37, d (7.9)   | 5.37, d (7.9)     |
| OH-9     | 3.25, br s      | 3.44, d (8.0)     | 3.29, s         | 3.29, s           | 3.29, s         | 3.29, s           |
| OH-12    | 21.3, CH_3      | 2.03, s           | 1710.4, qC      | 1.93, s           | 1710.4, qC      | 1.93, s           |

\(^{a}\)Recorded at 100 MHz. \(^{b}\)Recorded at 500 MHz.
Table 3: NMR spectroscopic data for punctaporins R (5) and S (6) in acetone-\(d_6\).

| Position | \(\delta_C\)^{a, mult.} | \(\delta_H\)^{b (J in Hz)} | \(\delta_C\)^{c, mult.} | \(\delta_H\)^{b (J in Hz)} |
|----------|--------------------------|-----------------------------|--------------------------|-----------------------------|
| 1        | 51.2, qC                 |                              | 53.6, qC                 |                              |
| 2        | 43.8, CH                 | 2.09, t (7.3)               | 39.2, CH                 | 2.01, dd (12.4, 7.8, 1.4)    |
| 3a       | 35.8, CH                 | 2.02, dd (7.3, 5.5)         | 37.8, CH                 | 2.37, dd (12.4, 9.2)         |
| 3b       | 1.54, t (5.5)            |                              | 1.73, dd (9.2, 7.8)      |                              |
| 4        | 42.1, qC                 |                              | 42.2, qC                 |                              |
| 5        | 80.2, qC                 |                              | 82.6, qC                 |                              |
| 6        | 69.3, CH                 | 3.47, dd (11.3, 7.3)        | 69.0, CH                 | 3.66, dd (10.2, 5.5)         |
| 7a       | 42.9, CH                 | 1.71, d (11.3)              | 42.4, CH                 | 1.94, dd (12.2, 5.5)         |
| 7b       | 1.54, t (5.5)            |                              | 1.86, dd (12.2, 10.2)    |                              |
| 8        | 53.6, qC                 |                              | 48.8, qC                 |                              |
| 9        | 149.2, CH                | 5.85, d (5.8)               | 150.0, CH                | 6.01, d (5.7)                |
| 10       | 126.8, CH                | 5.82, dd (5.8, 2.2)         | 126.8, CH                | 5.70, dd (5.7, 2.7)          |
| 11       | 90.9, CH                 | 3.62, d (2.2)               | 81.3, CH                 | 5.28, d (2.7)                |
| 12a      | 57.3, CH                 | 3.65, d (11.4)              | 63.6, CH                 | 4.88 d (10.2)                |
| 12b      | 4.06, dd (11.4, 7.0)     |                              | 4.06, dd (10.2, 1.4)     |                              |
| 13       | 23.3, CH                 | 1.10, s                     | 22.9, CH                 | 1.14, s                     |
| 14       | 24.2, CH                 | 1.08, s                     | 23.6, CH                 | 1.10, s                     |
| 15       | 27.3, CH                 | 1.18, s                     | 279, CH                  | 1.11, s                     |
| 16       | 60.7, CH                 | 3.19, s                     |                         |                              |
| OH-5     |                          | 5.29, br s                  |                         |                              |
| OH-6     |                          | 5.18, d (7.3)               |                         | 3.69, (5.5)                  |
| OH-12    |                          | 3.09, d (7.0)               |                         |                              |
| Ac-11    |                          | 20.8, CH                   | 1.93, s                  |                              |
| Ac-12    |                          | 21.0, CH                   | 170.1, qC                |                              |

^{a}Recorded at 150 MHz. {^{b}Recorded at 500 MHz. {^{c}Recorded at 100 MHz.

Figure 2: Key NOESY correlations for punctaporin N (1).

were nearly identical to those of the coisolated known compound 6-hydroxy-punctaporin A (8) [17], except that the exchangeable proton at 4.18 ppm (OH-9) was replaced by a methyl group (\(\delta_H/\delta_C\) 3.33/57.5). An HMBC correlation from the O-methyl proton signal (H\(_3\)-16) to the oxymethine carbon at 97.3 ppm (C-9) suggested that 2 is the C-9 methyl ether of 8. The absolute configuration of 2 was proposed as shown by analogy to 8, which was also supported by the nearly identical specific rotation values recorded for both compounds [17].

The molecular formula of punctaporin P (3) was determined to be C\(_{17}\)H\(_{26}\)O\(_5\) (five degrees of unsaturation) by HRESIMS, which is 28 mass units more than that of 2, corresponding to an extra carbonyl group. The NMR data of 3 (Table 2) were similar to those of 2, except that the oxymethine proton at 3.60 ppm (H-9) was significantly downfield to 5.11 ppm in 3. In addition, the O-methyl group attached to C-9 was replaced by an acetyl unit (\(\delta_H/\delta_C\) 2.03/21.3, 170.4), indicating that the C-9 oxygen of 3 was acetylated. An HMBC correlation from the downfield oxymethine proton (H-9) to the carboxylic carbon at 170.4 ppm indicated that 3 is the C-9 monoacetate of 8, with its configuration similarly deduced by analogy to compounds 1 and 2.

Punctaporin Q (4) gave a pseudomolecular ion [M + Na]+ peak by HRESIMS, consistent with the molecular formula C\(_{17}\)H\(_{26}\)O\(_5\) (five degrees of unsaturation), which is the same as 3. Its NMR spectra showed resonances similar to those of 3, indicating that 4 is also a monoacetate of 8, but with a different position for acetylation. Specifically, the \(^1\)H NMR chemical shifts of H\(_2\)-12 in 4 (\(\delta_H\) 4.37 and 4.57,
Table 2) were significantly downfield compared to those in 3 (δ_H 3.48 and 3.73), suggesting that OH-12 was acetylated. The observation was supported by an HMBC correlation from H_2-12 to the carboxylic carbon of the acetyl unit (δ_C 171.0).

The elemental composition of punctaporonin R (5) was established as C_{16}H_{26}O_4 (four degrees of unsaturation) by HRESIMS, which has one more CH_2 unit than another coisolated known compound, 6-hydroxypunctaporonin E (9) [17]. Comparison of the NMR data of 5 (Table 3) and 9 readily identified 5 as the C-11 methyl ether (δ_H/δ_C 3.19/60.7) of the latter and having the same configuration as its precedent.

Punctaporonin S (6) was assigned the molecular formula C_{19}H_{30}O_6 (six degrees of unsaturation) by HRESIMS. Its NMR spectra showed structural characteristics similar to those of 5, but the 1H NMR chemical shifts of H-11 (δ_H 5.70) and H_2-12 (δ_H 4.06 and 4.88) were significantly downfield compared to 5 (H-11: δ_H 3.62; H_2-12: δ_H 3.65 and 4.06), indicating that both OH-11 and OH-12 are acetylated, which were supported by relevant HMBC correlations. Therefore, 6 was assigned as the diacetate of the known compound 9, with its configuration deduced as shown.

To verify that the new metabolites 1–6 are authentic natural products, a portion of the freeze-dried fermented rice substrate was extracted with distilled, HPLC grade acetone, and the resulting extract was subjected to RP HPLC analysis using distilled, HPLC grade H_2O and MeOH as solvents. Compounds 1–6 were identified on the HPLC chromatogram of the crude extract by comparison of their retention times with the pure compounds, indicating that 1–6 are indeed naturally occurring metabolites.

Compounds 2, 5, and 6 showed modest cytotoxicity against HeLa (cervical epithelium) cells, showing IC_{50} values of 16.6, 10.4, and 47.4 μM, respectively, while the positive control cisplatin showed an IC_{50} value of 7.6 μM. The remaining compounds did not show noticeable cytotoxic effects against HeLa cells (IC_{50} > 100 μM).

4. Conclusion

In summary, six new caryophyllene sesquiterpenoids, punctaporonins N–S (1–6), and three known ones, 6-hydroxypunctaporonins B (7), A (8), and E (9), have been isolated from solid cultures of Cytospora sp. The structures of 1–6 were elucidated primarily by NMR spectroscopy. The absolute configuration of 1 was assigned by X-ray crystallographic analysis of its S-MTPA ester. Compounds 2, 5, and 6 showed modest cytotoxicity against HeLa cells. These results implied that the fungi isolated from those unique and competitive niches could prove to be valuable sources of new bioactive natural products.

Conflicts of Interest

The authors have declared that there are no conflicts of interest.

Acknowledgments

The authors gratefully acknowledge financial support from the National Natural Science Foundation of China (81202416).

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Crystallographic data for compound 1a have been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC778343). Copies of the data can be obtained, free of charge, on application to the director, CCDC 12 Union Road, Cambridge CB2 1EZ, UK, 2017.

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