New Punctaporonins from Two Fungicolous Isolates of

***Pestalotiopsis* Sp.**

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ABSTRACT

Seven new caryophyllene sesquiterpenoids (**5** – **11**) were isolated during investigation of two fungicolous isolates of *Pestalotiopsis* sp. and identified as punctaporonin analogues. The structures of these compounds were mainly assigned by analysis of NMR and HRMS data. Compounds **5**, **6**, and **7** are 13-hydroxy analogues of 6-hydroxypunctaporonins A, B, and E, respectively, and compound **8** is a 6-hydroxy analogue of punctaporonin D. Compounds **9** and **11** are ketone-containing analogues of 6-hydroxypunctaporonin A and fuscoatrol A, respectively, and compound **10** is a 9-methoxy derivative of **8**. The names punctaporonins N, O, and P are proposed for compounds **9**, **10**, and **11**, respectively. Antibacterial activity associated with the extract was attributed to the presence of known punctaporonins as major metabolites.

Keywords:

Caryophyllene; Sesquiterpenoids; Fungicolous; Fungi; *Pestalotiopsis*; Punctaporonins

1. Introduction

Caryophyllene and various analogues and derivatives thereof have been isolated from both plants and fungi (Collado et al., 1998). A characteristic feature of caryophyllene-related structures is a bicyclic ring system consisting of *trans*-fused four- and nine-membered rings. Tricyclic ring systems are sometimes formed by cross-ring linkages of the nine-membered ring (Collado et al., 1998). In the late 1980's, a series of oxidized caryophyllene analogues was reported from cultures of the fungus *Poronia punctata*. The first members of the series were initially referred to as punctatins, but the names were later changed to punctaporonins (Anderson et al., 1988; Poyser et al., 1986). Since then, numerous related, oxidized caryophyllene-type sesquiterpenoids have been reported from fungi, including 6-hydroxypunctaporonins and pestalotiopsins from *Pestalotiopsis* spp. (Deyrup et al., 2006; Pulici et al., 1996), fuscoatrol A from *Humicola fuscoatra* (Smetanina et al., 2004), Sch 725432, Sch 601253, Sch 601254, and Sch 725434 from *Chrysosporium pilosum* (Yang et al., 2009), cytosporinols from *Cytospora* sp. (Li et al., 2012), and punctaporonins H – M from *Hansfordia sinuosae* (Wu et al., 2014). Despite the unique and diverse chemistry associated with these compounds, their biological properties have been of limited interest to date, though some pestalotiopsins show immunosuppressive and cytotoxic activities and punctaporonin K reduces triglycerides and total cholesterol levels in cells (Pulici et al., 1996; Wu et al., 2014; Takao et al., 2009).

In the course of our studies of fungicolous fungi as sources of new bioactive natural products (Deyrup et al., 2006; Gloer, 2007; Hwang et al., 2015; Shim et al., 2011), the ethyl acetate extract of cultures of a fungicolous isolate of *Pestalotiopsis* sp. (MYC–

709) obtained from the surface of a polypore collected in Georgia, USA showed antimicrobial activity. Chemical investigation of the extract resulted in discovery of pestaloporonins A – C, three related sesquiterpenoids containing new ring systems and/or skeletons (Hwang et al., 2015). However, NMR data revealed the extract to be exceptionally rich in terpenoid metabolites, suggesting that more in-depth studies might be fruitful. Further studies of this isolate afforded four known caryophyllene sesquiterpenoids (**1** – **4**) and seven new analogues (**5** – **11**). Details regarding the isolation and structure elucidation of these metabolites are described here. Upon completion of this work, efforts to identify this *Pestalotiopsis* isolate to species were met with the discovery that the corresponding stored cultures were no longer viable. However, similarities in the metabolites encountered with those reported earlier by our group from another fungicolous isolate of *Pestalotiopsis* (*P. disseminata* MYC-1444 = NRRL 36915; deposited in the NRRL collection at the USDA National Center for Agricultural Utilization Research) led us to analyze the latter for the presence of the new compounds described above. Four of the same new compounds (**6** – **9**) were found in the corresponding extract, along with the previously reported compounds **1** – **3** (Deyrup et al., 2006). Minor peaks that could correspond to **4**, **5**, **10**, and **11** were also present, but the complexity of the extract precluded definitive detection. These observations indicate that *Pestalotiopsis* sp. MYC-709 was very likely an isolate of *P. disseminata*, but more importantly, that most, if not all, of the same compounds could be obtained from a culture that is viable and accessible in the NRRL collection.

2. Results and discussion

The acetonitrile-soluble portion of the crude ethyl acetate extract from solid-substrate fermentation cultures of *Pestalotiopsis* sp. MYC-709 was fractionated using silica gel. Further purification with reversed-phase HPLC led to the isolation of new caryophyllene sesquiterpenoids **5** – **11**, together with the known metabolites, 6-hydroxypunctaporonins E (**1**), A (**2**), and B (**3**), and fuscoatrol A (**4**). Compounds **5**, **6**, and **7** are 13-hydroxy analogues of 6-hydroxypunctaporonins A, B, and E, respectively. Compound **8** is a 6-hydroxy analogue of punctaporonin D, and its 9-methoxy derivative (**10**) was also encountered. Compounds **9** and **11** are oxidized analogues of 6-hydroxypunctaporonin A and fuscoatrol A, respectively.

6-Hydroxypunctaporonin E (**1**) crystallized from methanol after HPLC purification. The ^1H NMR spectrum showed signals typical of punctaporonins, including distinctive *cis*-coupled ($J = 5.6$ Hz) olefinic protons (H-9 and H-10), an isolated oxygenated methylene unit (H₂-12), and three methyl singlets (H₃-13, H₃-14, and H₃-15). In addition, two oxygenated methine signals (H-6 and H-11) were observed. Comparison of the ^1H NMR and specific rotation data with those of an authentic standard enabled identification of this compound as **1** (Deyrup et al., 2006). Compounds **2** – **4** were similarly identified by NMR and MS comparison to available standards and/or literature data (Deyrup et al., 2006; Pulici et al., 1996; Smetanina et al., 2004). Fuscoatrol A (**4**) lacks a ring closure characteristic of the punctaporonins, and appears in solution as a 3:1 equilibrium mixture of two conformational isomers. This observation is consistent with the literature report of **4** (Smetanina et al., 2004).

HRESIMS analysis of new compound **5** gave a pseudomolecular ion at m/z 283.1542 $[M - H]^-$ corresponding to the molecular formula $C_{15}H_{24}O_5$ (four degrees of unsaturation). The 1H NMR spectrum closely resembled that of **2**, including fortuitously coincident signals for two olefinic protons, an isolated oxygenated methylene, and two oxygenated methines. The relatively small (9.6 Hz) geminal coupling between the two C-3 protons was consistent with retention of the cyclobutane unit (Deyrup et al., 2006). However, the data for **5** (Table 1) displayed one less methyl singlet, instead showing an oxygenated methylene signal at δ_H 3.67 (H₂-13), suggesting replacement of one of the methyl groups of **2** with a CH₂OH unit. This hypothesis was consistent with the presence of one additional oxygen atom in the molecular formula relative to that of **2**, and was supported by analysis of ^{13}C NMR and HSQC data, which showed the presence of a disubstituted olefin unit, one oxygenated quaternary carbon, two oxygenated methine carbons, and two oxygenated methylene carbons. The additional hydroxy group relative to **2** was placed at C-13 by analysis of NOESY data (see below) and HMBC correlations from H₃-14 to C-13 as well as from H₂-13 to C-3, C-4, C-5, and C-14. The remainder of the structure was confirmed to match that of **2** on the basis of HMBC correlations summarized in Figure 1.

The NOESY data for **5** (Fig. 2) were analogous to those of **2** (Deyrup et al., 2006), except that correlations of new oxymethylene protons H₂-13 with H-2, H-3b, and H-6 indicated that this hydroxymethylene group (CH₂-13) is oriented as shown. Correlations of H-12a with H-3a and H₃-15, and of H₃-15 with H-9 placed all of these hydrogens on the opposite face of the ring system relative to CH₂-13. Because all of the previously known 6-hydroxypunctaporonin derivatives were also encountered in this extract, the

absolute configuration of **5** was assumed to match the relevant features of 6-hydroxypunctaporonin A (1*S*, 2*R*, 5*S*, 6*S*, 8*S*, 9*R*) (Deyrup et al., 2006). The new stereogenic center at C-4 was assigned the *R*-configuration based on the relative configuration assigned by interpretation of the NOESY data. The trivial name 6,13-dihydroxypunctaporonin A was proposed for compound **5**.

Compound **6** was recognized as an isomer of **5** on the basis of HRESIMS data. Furthermore, the ¹H and ¹³C NMR data for **6** were very similar to those for **3**, with the only evident difference being replacement of one of the methyl singlets with an AB pattern characteristic of an oxygenated CH₂ unit, indicating replacement of one of the geminal methyls of **3** with an isolated CH₂OH group, as was the case for **5**. As expected, relevant HMBC correlations from H₃-14 to C-13 and from H₂-13 to C-3, C-4, C-5, and C-14 again located the new hydroxy group at C-13, thereby establishing the gross structure of **6** as shown.

The relative configuration of **6** was determined to be analogous to that of **3** by analysis of NOESY data (Deyrup et al., 2006). Specifically, correlations of H-2 with H-6 and H-10, and of H₃-15 with H-11 indicated **6** that possesses the same *trans*-fused bicyclic system with analogous dispositions of the substituents of the nine-membered ring (Deyrup et al., 2006). The *E*-configuration of the C-1/C-11 olefin was confirmed by a cross-peak between H-12b and H-11. In addition, the *J*_{H9-H10} value (*J* = 13 Hz) is consistent with the literature report of **3**, supporting the *Z*-configuration for the C-9/C-10 olefin (Deyrup et al., 2006). As was the case for **5**, H₂-13 correlated to H-2 and H-6, placing the hydroxylated CH₂-13 on the same face of the ring system with these hydrogen atoms, leading to the identification of **6** as 6,13-dihydroxypunctaporonin B. The absolute

configuration is proposed to be analogous to that previously assigned for **3** (Deyrup et al., 2006).

^1H and ^{13}C NMR data for compound **7** resembled those of **1**, including the characteristic 6 Hz J -value for the *cis*-olefinic protons. HRESIMS data indicated that **7** is an isomer of the dihydroxylated punctaporonin analogues **5** and **6**. Again, as was the case for **5** and **6**, HMBC data revealed that C-13 was hydroxylated. In addition, HMBC correlations of H₃-15 with olefinic carbon C-9 and of H-2 with oxygenated methine carbon C-11 showed that **7** is a regioisomer of **5**, in which the allylic alcohol unit is transposed from its position in **5**. The NOESY data for **7** were analogous to those of **1**. H₂-13 again correlated to H-2 and H-6, establishing the relative orientation of the new CH₂OH unit as shown in **7**. The absolute configuration was presumed to be analogous to that of **1** (Deyrup et al., 2006), and this metabolite was given the name 6,13-dihydroxypunctaporonin E.

Compound **8** is another 6-hydroxypunctaporonin isomer. The ^1H NMR spectrum of **8** was nearly identical to that of **2**, except for a significant downfield shift in the signal for H-9, and slightly upfield shifts in the signals for H-2 and H-6. HMBC data revealed the connectivity of **8** to be identical to that of **2**. However, NOESY correlations of H-9 with H-2 and H-6 showed that the relative configuration of **8** at C-9 is inverted relative to that of **2** (Fig. 3). This stereochemical difference was consistent with a significant difference in the specific rotation observed for **8** (+104; c 0.15, MeOH) relative to that of **2** (-11; c 0.07, MeOH; Deyrup et al., 2006). Thus, **8** differs from the known compound punctaporonin D only by addition of a hydroxy group at C-6 (Anderson et al., 1988; Poyser et al., 1986). It was therefore assigned the trivial name 6-hydroxypunctaporonin

D. Recently, the 6-*O*-acetyl analogue of this compound was reported from the sponge-associated fungus *Hansfordia sinuosae* and assigned the name punctaporonin M (Wu et al., 2014). The absolute configuration was presumed to be analogous to that of **2** except for the inverted stereocenter at C-9 (Deyrup et al., 2006).

The molecular formula of **9** was assigned as C₁₅H₂₂O₄ on the basis of HRESIMS data. The NMR data for **9** displayed close similarity with those of the 6-hydroxypunctaporonins except for changes in the signals corresponding to positions 9, 10, and 11 (Tables 1 and 2), which suggested the presence of an α,β -unsaturated ketone moiety rather than an allylic alcohol unit. The presence of such a unit was consistent with the UV spectrum, and with the five degrees of unsaturation indicated by the molecular formula. Key HMBC correlations from H₃-15 to ketone carbon C-9 and from H₂-12 to olefinic carbon C-11 enabled assignment of the regiochemistry of the α,β -unsaturated ketone moiety as shown in **9**. Oxidation of a sample of **2** using PCC and pyrazole afforded a product with NMR data identical to those of **9** (Parish et al., 1984). Therefore, the relative and absolute configurations were assigned to match those of **2** (Deyrup et al., 2006). The name punctaporonin N was assigned to compound **9**.

Compound **10** was determined to have the molecular formula C₁₆H₂₆O₄ on the basis of HRESIMS data. The ¹H NMR spectrum of **10** was nearly identical to those of **8** and **2**, except for the presence of a signal for a methoxy group. HMBC data revealed that the connectivity of **10** is identical to that of **8** and **2** except for the linkage of the methoxy group to C-9. The relative configuration of **10** was assigned to match that of **8** by analysis of NOESY data including correlations of H-2 with H-3b, H-6, and H-9; of H-6 with H-9; of H-3a with H-12a; and of H-12b with H₃-15. Also, as was the case for **8**, the

specific rotation value for **10** was positive, in contrast to the negative value observed for **2**. The absolute configuration was presumed to be analogous to that of **8** (Deyrup et al., 2006). The name punctaporonin O was proposed for compound **10**.

Compound **11** gave the molecular formula $C_{16}H_{24}O_4$ by HRESIMS analysis. The 1H NMR data for **11** showed close resemblance to the set of signals corresponding to the minor solution conformer of **4**. Replacement of one oxygenated methine unit in structure **4** with a ketone group was suggested by changes in the 1H and ^{13}C NMR spectra (Tables 1 and 2). Analysis of HSQC and HMBC data, including HMBC correlations from H-2 and H₂-7 to ketone carbon C-6, indicated that **11** differs from **4** by oxidation of the C-6 secondary alcohol group to a ketone unit. The relative configuration of **11**, including *E*-geometry for both olefin units, was found to be analogous to that of **4** by analysis of NOESY data. Interestingly, in spite of the similarity to **4**, the 1H NMR spectrum of **11** showed only one conformer in solution. NOESY correlations of H₃-15 with H-2, H-7b, and H-10 were consistent with adoption of a conformation most closely resembling the minor conformer of **4** (Pulici et al., 1996; Smetanina et al., 2004). A recently published enantioselective total synthesis of pestalotiopsin A (Takao et al., 2009) enabled presumption of the absolute configuration of related compound **4** (Pulici et al., 1996; Smetanina et al., 2004). The absolute configuration of **11** is presumed to be analogous to that of **4**. The 6-*O*-acetyl-10-hydroxy analogue of fuscoatrol A has been named punctaporonin I (Wu et al., 2014). Therefore, the name punctaporonin P is suggested here for **11**. Neither **10** nor **11** were exposed to methanol during the isolation process, and are therefore considered unlikely to be artifacts arising from the process.

Compounds **5** – **11** were tested for antifungal activity against *Aspergillus flavus* and *Fusarium verticillioides*, but did not account for the activity originally observed for the ethyl acetate extract. Compounds **1** and **3** are known to show antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* (Deyrup et al., 2006), and **1** was a major constituent of the extract, however, compounds **5** – **11** were inactive in disk assays (Bauer et al., 1966; Wagenaar et al., 2001) towards *S. aureus*, *B. subtilis*, *Escherichia coli*, and *Candida albicans* at 100 µg/disk.

3. Experimental

3.1. General experimental procedures.

Optical rotations were measured on an AUTOPOL[®] III automatic polarimeter (Rudolph Research Analytical, Hackettstown, NJ). UV data were obtained using MeOH solutions with a Varian Cary III UV/vis spectrophotometer. HPLC separations were carried out using a Beckman System Gold instrument with a model 166P variable-wavelength UV detector connected to a 128 solvent module, equipped with either a preparative Dynamax C18 column (Rainin, 2.0 × 30 cm, 8 µm) or semi-preparative Apollo C18 column (Alltech, 1.5 × 25 cm, 5 µm) under UV detection at 210 nm. ¹H NMR data measurements were conducted using Bruker AVANCE-400 or AVANCE-600 spectrometers. ¹³C (150 MHz) and 2D (COSY, HSQC, HMBC, NOESY) NMR spectra were recorded on the Bruker AVANCE-600 spectrometer. ¹³C NMR multiplicities were not directly measured, but all assignments are fully consistent with HMQC data. All NMR experiments were performed at 294 K, using CDCl₃ or methanol-*d*₄ as the solvent. Chemical shifts were referenced to residual solvent signals for CDCl₃ (δ_H/δ_C, 7.26/77.2)

or methanol- d_4 (δ_H/δ_C , 3.31/49.0). NMR data processing was carried out with MestReNova v6.0.2 software. HRESITOFMS data were obtained on a Waters Q-ToF Premier mass spectrometer.

3.2. Fungal culture and extract

Pestalotiopsis sp. MYC-709 was isolated from a basidioma of *Stereum complicatum* found on a dead hardwood branch in FDR State Park, Warm Springs, Georgia, USA. After chemical investigation as described below, efforts to identify this organism to species and deposit a subculture in the NRRL culture collection led to the realization that the stored cultures were no longer viable. However, analysis of extract fractions from another, already-deposited *Pestalotiopsis* culture in our laboratory from which punctaporonin analogues **1–3** had originally been isolated (*P. disseminata* NRRL 36915; Genbank deposition number GU183121.1; Deyrup et al., 2006; Hwang et al., 2015) revealed the presence of at least four of the same new analogues (**6–9**). NRRL 36915 also originated as a fungicolous isolate from Georgia, although from a different host and from a site 150 miles to the southeast (from stromata of an unidentified pyrenomycete growing on a dead hardwood branch in Reed Bingham State Park, Adel, GA). This result indicated that *Pestalotiopsis* sp. MYC-709 was likely to be *P. disseminata*, although this could not be definitively established. Thus, while the procedure described below was that used for the MYC-709 extract, the compounds above were also produced by cultivation of the *P. disseminata* NRRL 36915 culture deposited at the USDA NCAUR. Fermentation and extraction processes leading to production of the extract described below have been reported previously (Hwang et al., 2015).

3.3. Isolation

The EtOAc extract (687 mg) obtained from solid-substrate fermentation on rice was partitioned between hexane (3×8 mL) and MeCN (8 mL). The MeCN-soluble portion (388 mg) was subjected to silica gel column chromatography (3.2×16 cm) eluting with a stepwise gradient of hexane/EtOAc (200 mL each of 1:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:1), followed by pure MeOH (300 mL) resulting in nine combined fractions based on TLC data (Fr. 1 – 9). A portion of Fr. 7 (98 mg out of 143 mg) was applied to C₁₈ HPLC (2.0×30 cm, 8 mL/min) using 18% MeCN/H₂O for 40 min to yield major components **1** (15 mg, $t_R = 25.2$ min) and **2** (29 mg, $t_R = 35.2$ min). Fr. 9 (157 mg) was fractionated over C₁₈ HPLC (2.0×30 cm, 8 mL/min) with 15% MeCN/H₂O over 30 min to obtain **5** (Fr. 9-3; 2.0 mg, $t_R = 14.1$ min), **6** (Fr. 9-5; 2.4 mg, $t_R = 27.5$ min), and four other subfractions (Fr. 9-1, 9-2, 9-4, and 9-6). Fr. 9-2 (3.9 mg) was purified by C₁₈ HPLC (1.0×25 cm, 1.5 mL/min) with 12% MeCN/H₂O for 20 min to afford **7** (0.7 mg, $t_R = 16.0$ min). Fr. 8 (29 mg) was chromatographed over C₁₈ HPLC (2.0×30 cm, 8 mL/min) using 18% MeCN/H₂O for 40 min, followed by a linear increase of MeCN/H₂O from 18% to 60% over 20 min to give **8** (3.0 mg, $t_R = 39.1$ min) and **3** (6.7 mg, $t_R = 51.9$ min). Fr. 6 (26 mg) was subjected to C₁₈ HPLC (1.0×25 cm, 2 mL/min) with 28% MeCN/H₂O for 20 min, then a linear gradient of MeCN/H₂O from 28% to 50% over 45 min to yield **9** (1.1 mg, $t_R = 15.7$ min) and **10** (1.7 mg, $t_R = 37.0$ min). Compounds **4** (7.8 mg, $t_R = 32.1$ min) and **11** (1.4 mg, $t_R = 33.3$ min) were isolated from Fr. 5 (28 mg) by C₁₈ HPLC (1.0×25 cm, 2 mL/min) eluting with 30% MeCN/H₂O for 15 min, followed by a linear increase to 60% MeCN over 45 min. Known compounds **1** – **4** were identified

by comparison of their NMR and $[\alpha]_D$ data to literature values (Deyrup et al., 2006; Smetanina et al., 2004).

3.3.1. 6,13-Dihydroxypunctaporonin A (**5**)

Colorless glass; $[\alpha]_D^{22} -11$ (c 0.11, MeOH); ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HMBC data: H-2 $\rightarrow$ C-1, 3, 4, 5, 6, 11, 12; H-3a $\rightarrow$ C-1, 2, 4, 5, 13; H-3b $\rightarrow$ C-2, 4, 5, 6*, 13; H-6 $\rightarrow$ C-5, 7; H-7a $\rightarrow$ C-1, 5, 6, 8, 9; H-7b $\rightarrow$ C-5, 6, 8, 9, 15; H-9 $\rightarrow$ 1, 10, 11, 15; H-10 or 11 $\rightarrow$ C-1, 2, 8, 9, 10, 11, 12; H-12a $\rightarrow$ C-1, 2, 8, 11; H-12b $\rightarrow$ C-1, 2, 8, 11; H₂-13 $\rightarrow$ C-3, 4, 5, 14; H₃-14 $\rightarrow$ C-3, 4, 5, 13; H₃-15 $\rightarrow$ C-1, 6*, 7, 8, 9; *Four-bond correlation; NOESY data: H-2 $\leftrightarrow$ H-3b, 6, 13; H-3a $\leftrightarrow$ H-12a, 14; H-3b $\leftrightarrow$ H-2, 13; H-6 $\leftrightarrow$ H-2, 7a, 13; H-7a $\leftrightarrow$ H-6, 9; H-7b $\leftrightarrow$ H-15; H-9 $\leftrightarrow$ H-7a, 15; H-12a $\leftrightarrow$ H-3a; H-12b $\leftrightarrow$ H-15; H₂-13 $\leftrightarrow$ H-2, 3b, 6, 14; H₃-14 $\leftrightarrow$ H-3a, 13; H₃-15 $\leftrightarrow$ H-7b, 9, 12b; HRESIMS m/z 283.1542 $[\text{M} - \text{H}]^-$ (calcd for C₁₅H₂₃O₅, 283.1545).

3.3.2. 6,13-Dihydroxypunctaporonin B (**6**)

Colorless glass; $[\alpha]_D^{22} -80$ (c 0.12, MeOH); ^1H NMR data, see Table 1; ^{13}C NMR data, see Table 2; HMBC data: H-2 $\rightarrow$ C-1, 3, 5, 6, 11, 12; H-3a $\rightarrow$ C-1, 2, 4, 13, 14; H-3b $\rightarrow$ C-2, 4, 5, 13; H-6 $\rightarrow$ C-7, 8; H-7a $\rightarrow$ C-5, 6; H-7b $\rightarrow$ C-6, 9; H-9 $\rightarrow$ 7, 11; H-10 $\rightarrow$ C-1, 8; H-11 $\rightarrow$ C-1, 2, 9, 12; H-12a $\rightarrow$ C-1, 11; H-12b $\rightarrow$ C-1, 2, 11; H-13a $\rightarrow$ C-3, 4, 5, 14; H-13b $\rightarrow$ C-3, 4, 5, 14; H₃-14 $\rightarrow$ C-3, 4, 5, 13; H₃-15 $\rightarrow$ C-7, 8, 9; NOESY data: H-2 $\leftrightarrow$ H-3b, 6, 7a, 9, 10, 11, 13a; H-3a $\leftrightarrow$ H-12a, 12b, 14; H-3b $\leftrightarrow$ H-2, 13a, 13b; H-6 $\leftrightarrow$ H-2, 7a, 7b, 9, 13b; H-7a $\leftrightarrow$ H-2, 6, 11, 15; H-7b $\leftrightarrow$ H-6, 15; H-9 $\leftrightarrow$ H-2, 6, 15; H-10

$\leftrightarrow$ H-2; H-11 $\leftrightarrow$ H-2, 7a, 12b, 15; H-12a $\leftrightarrow$ H-3a; H-12b $\leftrightarrow$ H-3a, 11; H-13a $\leftrightarrow$ H-2, 3b; H-13b $\leftrightarrow$ H-3b, 6, 14; H₃-14 $\leftrightarrow$ H-3a, 13b; H₃-15 $\leftrightarrow$ H-7a, 7b, 9, 11; HRESIMS m/z 283.1541 $[M - H]^-$ (calcd for C₁₅H₂₃O₅, 283.1545).

3.3.3. 6,13-Dihydroxypunctaporonin E (7)

Colorless glass; $[\alpha]_D^{22}$ -48 (*c* 0.06, MeOH); ¹H NMR data, see Table 1; ¹³C NMR data, see Table 2; HMBC data: H-2 $\rightarrow$ C-1, 3, 6, 11, 12; H-3a $\rightarrow$ C-2, 4, 13, 14; H-3b $\rightarrow$ C-2, 4, 5, 13; H-6 $\rightarrow$ C-5, 7; H-7a $\rightarrow$ C-6, 8, 9, 15; H-7b $\rightarrow$ C-1, 5, 6, 8, 9; H-9 $\rightarrow$ 1, 7, 8, 10, 11; H-10 $\rightarrow$ C-1, 8, 9, 11; H-11 $\rightarrow$ C-2, 8, 9, 10; H-12a $\rightarrow$ C-1, 2, 11; H-12b $\rightarrow$ C-2, 8; H₂-13 $\rightarrow$ C-3, 4, 5, 14; H₃-14 $\rightarrow$ C-3, 4, 5, 13; H₃-15 $\rightarrow$ C-1, 7, 8, 9; NOESY data: H-2 $\leftrightarrow$ H-3b, 6, 9, 10, 11, 13; H-3a $\leftrightarrow$ H-12b, 14; H-3b $\leftrightarrow$ H-2, 11; H-6 $\leftrightarrow$ H-2, 7b, 9, 13a; H-7b $\leftrightarrow$ H-6, 9; H-9 $\leftrightarrow$ H-2, 6, 7b, 10, 15; H-10 $\leftrightarrow$ H-2, 9; H-11 $\leftrightarrow$ H-2, 3b; H-12a $\leftrightarrow$ 15; H-12b $\leftrightarrow$ H-3a; H₂-13 $\leftrightarrow$ H-2, 6; H₃-14 $\leftrightarrow$ H-3a; H₃-15 $\leftrightarrow$ H-9, 12a; HRESIMS m/z 283.1533 $[M - H]^-$ (calcd for C₁₅H₂₃O₅, 283.1545).

3.3.4. 6-Hydroxypunctaporonin D (8)

White amorphous solid; $[\alpha]_D^{22}$ +104 (*c* 0.15, MeOH); ¹H NMR data, see Table 1; ¹³C NMR data, see Table 2; HMBC data: H-2 $\rightarrow$ C-1, 3, 5, 6, 11, 12; H-3a $\rightarrow$ C-2, 4, 13; H-3b $\rightarrow$ C-1, 2, 4, 5, 6*, 12*, 13; H-6 $\rightarrow$ C-5, 7; H-7a $\rightarrow$ C-1, 5, 6, 8, 9; H-7b $\rightarrow$ C-5, 6, 8, 9, 15; H-9 $\rightarrow$ C-1, 2*, 7, 10, 11, 12*, 15; H-10 $\rightarrow$ C-1, 8, 9, 11; H-11 $\rightarrow$ C-1, 2, 8, 9, 10, 12; H-12a $\rightarrow$ C-1, 2, 8, 11; H-12b $\rightarrow$ C-1, 2, 8, 11; H₃-13 $\rightarrow$ C-3, 4, 5, 14; H₃-14 $\rightarrow$ C-3,

4, 5, 13; H₃-15 → C-1, 6^{*}, 7, 8, 9; ^{*}Four-bond correlation; NOESY data: H-2 ↔ H-3b, 6, 9, 13; H-3a ↔ H-12a, 14; H-3b ↔ H-2, 13; H-6 ↔ H-2, 7a, 9, 13; H-7a ↔ H-6, 9, 15; H-7b ↔ H-6, 15; H-9 ↔ H-2, 6, 7a; H-12a ↔ H-3a; H-12b ↔ H-15; H₃-13 ↔ H-2, 3b, 6; H₃-14 ↔ H-3a; H₃-15 ↔ H-7a, 7b, 12b; HRESIMS *m/z* 267.1590 [M – H][–] (calcd for C₁₅H₂₃O₄, 267.1596).

3.3.5. *Punctaporonin N (9)*

White amorphous solid; [α]²²_D +18 (*c* 0.10, MeOH); UV (MeOH) λ_{max} 232 nm (log ε 3.35); ¹H NMR data, see Table 1; ¹³C NMR data, see Table 2; HMBC data: H-2 → C-1, 3, 4, 5, 6, 11, 12; H-3a → C-1, 2, 4, 14; H-3b → C-2, 4, 5, 6^{*}, 14; H-6 → C-5, 7; H-7a → C-1, 5, 6, 8, 9; H-7b → C-6, 8, 9, 15; H-10 → C-1, 2^{*}, 8, 9, 11; H-11 → C-1, 2, 8, 9, 10, 12; H-12a → C-1, 2, 8, 11; H-12b → C-1, 2, 8, 11; H₃-13 → C-3, 4, 5, 14; H₃-14 → C-3, 4, 5, 13; H₃-15 → C-1, 6^{*}, 7, 8, 9; ^{*}Four-bond correlation; HRESIMS *m/z* 265.1448 [M – H][–] (calcd for C₁₅H₂₁O₄, 265.1440).

3.3.6. *Punctaporonin O (10)*

White amorphous solid; [α]²²_D +42 (*c* 0.14, MeOH); ¹H NMR data, see Table 1; ¹³C NMR data, see Table 2; HMBC data: H-2 → C-3, 4, 5, 6, 11, 12; H-3a → C-1, 2, 4, 13, 14; H-3b → C-2, 4, 5, 6^{*}, 13, 14; H-6 → C-5, 7; H-7a → C-1, 5, 6, 9; H-7b → C-6, 8, 9, 15; H-9 → C-7, 10, 11, 15, OCH₃; H-10 → C-1, 2^{*}, 8, 9, 11; H-11 → C-1, 8, 9, 10, 12; H-12a → C-2, 8, 11; H-12b → C-1, 2, 11; H₃-13 → C-3, 4, 5, 14; H₃-14 → C-3, 4, 5, 13; H₃-15 → C-1, 6^{*}, 7, 8, 9; OCH₃ → C-9; ^{*}Four-bond correlation; NOESY data: H-2 ↔ H-3b, 6, 9, 10, 11, 13; H-3a ↔ H-12a, 14; H-3b ↔ H-2, 11, 13; H-6 ↔ H-2, 7a, 9, 13; H-7a

↔ H-6, 9; H-7b ↔ H-15; H-9 ↔ H-2, 6, 7a, 10; H-10 ↔ H-2, 9; H-11 ↔ H-2, 3b, 12a, 12b; H-12a ↔ H-3a, 11; H-12b ↔ H-11, 15; H₃-13 ↔ H-2, 3b, 6; H₃-14 ↔ H-3a; H₃-15 ↔ H-7b, 12b; OCH₃ ↔ H-9, 10; HRESIMS m/z 281.1744 [M – H][–] (calcd for C₁₆H₂₅O₄, 281.1753).

3.3.7. *Punctaporonin P (II)*

Colorless glass; $[\alpha]_D^{22}$ –52 (*c* 0.06, MeOH); ¹H NMR data, see Table 1; ¹³C NMR data, see Table 2; HMBC data: H-2 → C-1, 3, 5, 6, 11, 12; H-3a → C-1, 2, 4, 13, 14; H-3b → C-2, 4, 5, 6^{*}, 13; H-7a → C-5, 6, 8, 9; H-7b → C-5, 6, 8, 9, 10^{*}, 15; H-9 → 7, 11, 15; H-10 → C-1, 8, 9, 11, OCH₃; H-11 → C-1, 2, 5^{*}, 9, 12; H-12a → C-1, 2, 11; H-12b → C-1, 2, 11; H₃-13 → C-3, 4, 5, 14; H₃-14 → C-3, 4, 5, 13; H₃-15 → C-7, 8, 9; OCH₃ → C-10; ^{*}Four-bond correlation; NOESY data: H-2 ↔ H-3b, 10, 11, 13, 15; H-3a ↔ H-12a, 14; H-3b ↔ H-2, 13; H-7a ↔ H-9; H-7b ↔ H-13, 15; H-9 ↔ H-7a, 10, 11, 16; H-10 ↔ H-2, 9, 11, 15, 16; H-11 ↔ H-2, 9, 10, 12b, 16; H-12a ↔ H-3a; H-12b ↔ H-11; H₃-13 ↔ H-2, 3b, 7b; H₃-14 ↔ H-3a; H₃-15 ↔ H-2, 7b, 10; OCH₃ ↔ H-9, 10, 11; HRESIMS m/z 303.1563 [M + Na]⁺ (calcd for C₁₆H₂₄O₄Na, 303.1572), 583.3242 [2M + Na]⁺ (calcd for C₃₂H₄₈O₈Na, 583.3247).

3.4. *Oxidation of 6-hydroxypunctaporonin A.*

A sample of 6-hydroxypunctaporonin A (**2**; 5.0 mg) was dissolved in dry acetone (1.6 mL) with excess pyrazole (2%, 32 mg), and PCC (12.1 mg) was added to the solution. The reaction was stirred under argon at 2–3°C and monitored by TLC using 2,4-dinitrophenylhydrazine staining. After stirring for 4 hr, saturated aqueous NaCl

solution (10 mL) was added, and the mixture was extracted with CHCl_3 (3×10 mL). The organic layer was collected, dried, evaporated, and purified by C_{18} HPLC (2.0×30 cm, 8 mL/min) eluting with 22% MeCN/ H_2O for 20 min, followed by a linear increase from 22% to 50% MeCN/ H_2O over 20 min to obtain a sample of **9** (0.8 mg, $t_{\text{R}} = 24.0$ min), which gave NMR data identical to those of the sample of **9** isolated from the extract.

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Table 1.¹H NMR Spectroscopic Data (δ_{H} , J in Hz) for Compounds **5** – **11**

position	5^a	6^a	7^a	8^a	9^b	10^b	11^b
2	2.38, dd (13, 8.1)	3.36, dd (12, 8.6)	2.13, dd (13, 7.8)	2.04, dd (13, 5.4)	1.55, dd (12, 7.8)	2.03, dd (13, 7.2)	3.72, dd (11, 9.1)
3a	1.95, dd (13, 9.6)	2.08, dd (12, 11)	1.99, dd (13, 9.6)	2.08, dd (13, 6.0)	2.17, dd (12, 9.0)	2.09, dd (13, 8.4)	2.35, dd (11, 11)
3b	1.77, dd (9.6, 8.1)	1.56, dd (11, 8.6)	1.80, dd (9.6, 7.8)	1.48, dd (6.0, 5.4)	1.61, dd (9.0, 7.8)	1.52, dd (8.4, 7.2)	1.65, dd (11, 9.1)
6	4.11, dd (10, 5.4)	4.03, dd (4.3, 2.0)	3.51, dd (11, 4.8)	3.93, dd (11, 5.1)	3.64, dd (11, 5.4)	3.99, dd (11, 4.8)	
7a	1.89, dd (14, 5.4)	2.72, dd (16, 4.3)	1.83, dd (13, 11)	1.91, dd (13, 5.1)	2.41, dd (14, 5.4)	2.02, dd (14, 4.8)	3.68, ddd (19, 1.7, 1.5)
7b	1.67, dd (14, 10)	1.43, ddd (16, 2.0, 2.0)	1.72, dd (13, 4.8)	1.63, dd (13, 11)	1.68, dd (14, 11)	1.64, dd (14, 11)	3.00, d (19)
9	4.07, br s	5.73, ddd (13, 1.5, 1.5)	5.81, d (6.0)	4.86, s		4.36, dd (1.7, 1.2)	4.91, dm (10)
10	5.71, s	5.65, ddd (13, 2.4, 2.3)	5.63, dd (6.0, 2.7)	5.55, d (6.6)	6.00, d (6.0)	5.71, dd (6.1, 1.2)	4.61, br d (10)
11	5.71, s	5.87, br s	4.07, d (2.7)	5.54, d (6.6)	7.37, d (6.0)	5.53, dd (6.1, 1.7)	5.86, s
12a	3.70, d (11)	4.19, ddd (12, 1.7, 1.6)	4.06, d (11)	3.67, d (11)	3.85, d (11)	3.70, d (11)	4.04, d (11)
12b	3.48, d (11)	3.97, d (12)	3.69, d (11)	3.53, d (11)	3.69, d (11)	3.57, d (11)	3.85, d (11)
13a	3.67, s	3.91, d (11)	3.62, d (11)	1.20, s	1.14, s	1.21, s	1.19, s
13b	3.67, s	3.54, d (11)	3.59, d (11)				
14	1.18, s	1.19, s	1.18, s	1.13, s	1.10, s	1.14, s	1.00, s
15	1.02, s	1.22, s	1.25, s	0.99, s	1.22, s	1.04, s	2.01, dd (1.3, 1.2)
OCH ₃						3.47, s	3.27, s

^aData were collected at 600 MHz in methanol-*d*₄. ^bData were collected at 600 MHz in CDCl₃.

Table 2.¹³C NMR Spectroscopic Data (δ_C) for Compounds **5** – **11**

Position	5 ^a	6 ^a	7 ^{a,c}	8 ^a	9 ^b	10 ^{b,c}	11 ^{b,c}
1	55.8	139.4	50.6	55.7	54.5	54.0	136.0
2	48.5	40.4	43.5	46.6	50.5	45.3	40.6
3	31.6	30.6	31.4	36.0	35.2	34.9	33.9
4	48.6	46.5	47.7	43.5	42.7	42.6	42.6
5	80.1	82.6	79.7	80.0	79.9	79.2	87.4
6	70.8	73.9	70.5	69.2	68.3	68.2	208.0
7	40.6	45.4	43.0	40.1	37.8	40.1	50.1
8	50.8	74.5	53.5	54.5	56.3	54.0	132.5
9	88.3	142.8	148.3	82.4	213.1	90.8	132.5
10	130.7	124.9	129.1	132.1	128.0	127.7	75.2
11	142.1	126.8	81.9	137.6	166.6	137.1	135.0
12	62.5	65.0	61.3	63.4	60.9	62.7	68.5
13	67.1	68.3	66.9	23.5	23.7	23.1	26.2
14	19.5	19.9	19.3	24.3	23.1	23.5	21.5
15	24.6	32.1	27.4	17.6	23.8	17.0	19.8
OCH ₃						58.8	55.5

^aData were collected at 150 MHz in methanol-*d*₄. ^bData were collected at 150 MHz in CDCl₃.^cAssignments were made using HSQC and HMBC data collected at 600 MHz (¹H dimension).

1: R=H
7: R=OH

2: R₁, R₂=H; R₃=OH
5: R₁, R₃=OH; R₂=H
8: R₁, R₃=H; R₂=OH
9: R₁=H; R₂, R₃=O
10: R₁, R₃=H; R₂=OCH₃

3: R=H
6: R=OH

4: R₁=OH; R₂=H
11: R₁, R₂=O

Fig. 1. Key HMBC correlations for 6,13-dihydroxypunctaporonin A (**5**)

Fig. 2. Energy-minimized model and key NOESY correlations for 6,13-dihydroxypunctaporonin A (**5**)

Fig. 3. Energy-minimized model and key NOESY correlations for 6-hydroxypunctaporonin D (**8**)