Brasilamides A–D: Sesquiterpenoids from the Plant Endophytic Fungus *Paraconiothyrium brasiliense*


Keywords: Antiviral agents / Biological activity / Structure elucidation / Natural products / Configuration determination

New tricyclic sesquiterpenoids brasilamides A–D (1–4) and the known pinthunamide (5) have been isolated from cultures of the plant endophytic fungus *Paraconiothyrium brasiliense* Verkley. Their structures were elucidated primarily by NMR spectroscopy, and the structure of 1 was further confirmed by X-ray crystallography. The absolute configuration of the C-5 tertiary alcohol in 2 was assigned by analogy to 1 and confirmed by observing the circular dichroism of in situ generated [Rh2(OCOCF3)4] complex. Compounds 2–4 showed modest inhibitory effects on HIV-1 replication in C8166 cells. Compounds 1 and 2 possess an unprecedented 4-oxatricyclo-[3.3.1.02,7]nonane skeleton.

**Introduction**

Sesquiterpenoids incorporating the bergamotane skeleton have been reported from various natural sources. Examples of fungal metabolites include pinthunamide (5) isolated from *Ampulliferina* sp.,[1] ampullicin, and isampullicin, and dihydroampullicin from *Ampulliferina*-like sp. No 27,[2,3] bergamotene from *Aspergillus fumigatus*,[4] the expansolides from *Penicillum expansum* and *Aspergillus fumigatus* Fresenius,[5,6] the massarinolins from the aquatic fungus *Massarina tunicata*,[7] and the decipienolides from a coprophilous fungus *Podospora decipiens*.[8] Whereas those of plant metabolites include tanavulgarol from *Tanacetum vulgare*[9] and the clavigerins from the liverwort *Lepidolaena clavigera*.[10,11] Plant endophytic fungi are well-known sources of bioactive natural products,[12–16] and our previous chemical studies have also afforded a variety of bioactive secondary metabolites.[17–19] During our continuous search for new bioactive compounds from this class of fungi, a strain of *Paraconiothyrium brasiliense* Verkley (M3–3341), isolated from branches of *Acer truncatum* Bunge on Dongling Mountain, Beijing, P. R. China, was grown in a solid-substrate fermentation culture. An organic solvent extract of the culture showed an inhibitory effect on HIV-1 replication in C8166 cells. Fractionation of the extract afforded four new sesquiterpenoids, which we have named brasilamides A–D (1–4), and the known compound pinthunamide (5; Figure 1).[1] Details of the isolation, structure elucidation, and biological activity of these compounds are reported herein.

Figure 1. Metabolites 1–5 from *P. brasiliense*.

**Results and Discussion**

The molecular formula of brasilamide A (1) was established as C15H19NO5 (seven degrees of unsaturation) on the basis of its HRMS (ESI) spectrum (*m/z* = 316.1147 [M + Na]+, Δ = +0.8 mmu). Analysis of the 1H, 13C, and HMQC NMR spectroscopic data (Table 1) of 1 revealed three exchangeable protons, two methyl groups, three methylene units, three methines, two sp3 quaternary carbon atoms (one oxygenated), one trisubstituted olefin, one α,β-unsaturated ketone (δ = 200.0 ppm), and two carboxy (δ = 172.6 and 170.7 ppm) carbon atoms. Interpretation of the 1H–1H COSY NMR spectroscopic data established an isolated proton spin system corresponding to the C-6–C-10 (through C-7, C-8, C-1, and C-9) subunit. HMBC correlations from 1-H, 6-H, and 7-H to C-2 and C-5 indicate
that the C-5 oxygenated sp³ quaternary carbon (δ = 103.9 ppm) atom is located between C-6 and C-9, whereas the other sp³ quaternary carbon C-2 (δ = 45.9 ppm) atom is attached to both C-1 and C-7, completing the bicyclo-[3.1.1]heptane ring in I. Correlations from 11-H to C-1, C-2, C-3, C-7, and C-12 reveal the connection of C-2 to C-3 and C-11, and of the C-12 ketone carbon atom to C-11, whereas those from 13-H to C-12, C-14, C-15, and C-16 and from 16-H to C-13, C-14, and C-15 indicate that the C-13/C-14 olefin is conjugated to both the C-12 ketone and the C-15 carboxy carbon atoms. Considering the doubly oxygenated nature of C-5 (δ = 103.9 ppm) and the unsaturation requirement for I, the C-3 carboxy carbon atom must acylate one of the oxygen atoms attached to C-5 to form a δ-lactone moiety, thereby completing the 4-oxatricyclo-[3.3.1.0²,7]nonane skeleton in I. The remaining two exchangeable protons were assigned as 15-NH₂ by default. On the basis of these data, the gross structure of I was established, as shown in Figure 1.

Table 1. NMR spectroscopic data for I in [D₆]acetone.

<table>
<thead>
<tr>
<th>Pos.</th>
<th>δ_H [ppm] (mult., J [Hz])</th>
<th>δ_C [ppm][b]</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.20 (t, 6.0)</td>
<td>43.6, CH₂</td>
<td>2, 5, 7, 8, 9, 10, 11</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>45.9, C₅</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>172.6, C₅</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>103.9, C₅</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2.11 (br. d, 3.0)</td>
<td>41.7, CH₂</td>
<td>2, 5, 7, 8, 9</td>
</tr>
<tr>
<td>7</td>
<td>2.47 (m)</td>
<td>36.4, CH</td>
<td>1, 2, 5, 8, 11</td>
</tr>
<tr>
<td>8a</td>
<td>1.28 (d, 10)</td>
<td>36.4, CH₂</td>
<td>1, 2, 6, 7, 9</td>
</tr>
<tr>
<td>8b</td>
<td>2.67 (ddd, 10, 6.0, 5.5)</td>
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<tr>
<td>9</td>
<td>2.25 (ddd, 7.0, 6.0)</td>
<td>44.4, CH₂</td>
<td>1, 2, 5, 8, 10</td>
</tr>
<tr>
<td>10</td>
<td>0.96 (d, 7.0)</td>
<td>12.9, CH₁</td>
<td>1, 5, 9</td>
</tr>
<tr>
<td>11</td>
<td>3.14 (s)</td>
<td>44.1, CH₂</td>
<td>1, 2, 3, 7, 12</td>
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<tr>
<td>12</td>
<td></td>
<td>200.0, C₅</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>6.88 (d, 1.5)</td>
<td>129.3, CH</td>
<td>12, 14, 15, 16</td>
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<tr>
<td>14</td>
<td></td>
<td>145.0, C₅</td>
<td></td>
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<tr>
<td>15</td>
<td></td>
<td>170.7, C₅</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>2.16 (d, 1.5)</td>
<td>14.8, CH₂</td>
<td>13, 14, 15</td>
</tr>
<tr>
<td>OH-5</td>
<td>6.40 (br. s)</td>
<td></td>
<td></td>
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<tr>
<td>NH₂-15</td>
<td>6.58 (br. s); 7.16 (br. s)</td>
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</table>

[a] Recorded at 400 MHz. [b] Recorded at 100 MHz.

Ultimately, the structure of brasilamide A (I) was confirmed by single-crystal X-ray crystallographic analysis, and a perspective ORTEP plot is shown in Figure 2. The X-ray data allowed the relative configuration of brasilamide A to be determined as depicted in I. The presence of a relatively high percentage of oxygen in I should exhibit enough anomalous dispersion of Cu-Kα radiation to allow determination of its absolute configuration[20] with the Flack parameter value close to 0.0.[21] Therefore, the (1S,2S,5R,7R,9S) absolute configuration was proposed for I on the basis of the value of the Flack absolute structure parameter –0.02 (16).[21]

Brasilamide B (2) was assigned the elemental composition C₁₃H₂₃NO₃ (five degrees of unsaturation) by HRMS (ESI) analysis (m/z = 288.1559 [M + Na]⁺, Δ = +1.1 mmu), 28 mass units less than I. The 1H and 13C NMR spectra of 2 displayed signals for structural features similar to those found in I, except that the α,β-unsaturated ketone (C-12; δ

Figure 2. Thermal ellipsoid representation of I.

= 200.0 ppm) and the carboxy (C-3; δ = 172.6 ppm) carbon atoms in I are replaced by two methylenes (δ = 2.09/24.8 and 3.82, 3.86/69.4 ppm, respectively) in the spectra of 2. These observations were supported by HMBC correlations from 11-H to the methylene carbon atoms C-3 and C-12. The relative and absolute configurations of 2 were deduced to be the same as those of I by comparison of their 1H–1H coupling constants and NOESY data. As confirmation, the absolute configuration of the C-5 tertiary alcohol in 2 was also assigned on the basis of circular dichroism of the in situ formed [Rh₂(OCOCF₃)₄] complex,[22] with the inherent contribution subtracted. Upon addition of [Rh₂(OCOCF₃)₄] to 2 in CH₂Cl₂ solution, a metal complex of the C-5 tertiary alcohol with [Rh₂(OCOCF₃)₄] was generated as an auxiliary chromophore. It was demonstrated that the sign of the E band (at ca. 350 nm) can be used to correlate the absolute configuration of a tertiary alcohol by applying the bulkiness rule.[23] In this case, the Rh complex of 2 displayed a positive E band (Figure 3), correlating to the (5R) absolute configuration, which is consistent with that assigned for I.

Figure 3. CD spectrum of the in situ formed Rh complex of 2 with the inherent CD spectrum subtracted.

Brasilamide C (3) gives a pseudomolecular ion [M + Na]⁺ peak at m/z = 302.1359 (Δ = +0.4 mmu) in the HRMS (ESI) analysis, which corresponds to an elemental composition of C₁₃H₂₃NO₄ (six degrees of unsaturation). The 1H and 13C NMR spectroscopic data (Table 2) of 3 closely resemble those of known pinitunamide (5), except that the
Table 2. $^1$H and $^{13}$C NMR spectroscopic data of 2-4 in [D$_6$]acetone.

<table>
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<td>1.92 (t, 6.0)</td>
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<td>2</td>
<td>40.8, C$_q$</td>
<td>1.52 (m), 1.92 (m)</td>
<td>3</td>
<td>3.82 (d, 10), 3.86 (d, 10)</td>
<td>69.4, CH$_2$</td>
<td>4</td>
<td>2.18 (m)</td>
<td>41.1, CH$_2$</td>
</tr>
<tr>
<td>5</td>
<td>1.81 (d, 12), 2.17 (dd, 12, 6.5)</td>
<td>42.8, CH$_2$</td>
<td>7</td>
<td>2.23 (m)</td>
<td>38.4, CH</td>
<td>8</td>
<td>1.14 (d, 10), 2.46 (d, 10, 7.0, 6.0)</td>
<td>37.0, CH$_2$</td>
<td>9</td>
<td>1.89 (q, 7.0)</td>
<td>45.1, CH</td>
</tr>
<tr>
<td>10</td>
<td>1.02 (d, 7.0)</td>
<td>15.2, CH$_3$</td>
<td>11</td>
<td>1.64 (m)</td>
<td>22.8, CH$_2$</td>
<td>12</td>
<td>2.09 (m)</td>
<td>24.8, CH$_2$</td>
<td>13</td>
<td>6.38 (t, 6.5)</td>
<td>136.3, CH$_3$</td>
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<tr>
<td>15</td>
<td>1.80 (s)</td>
<td>12.7, CH$_2$</td>
<td>16</td>
<td>OH-5</td>
<td>4.68 (s)</td>
<td>OH-10</td>
<td>OH$_2$</td>
<td>3.33 (br. s)</td>
<td>17</td>
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<td></td>
<td></td>
<td>6.08 (br. s), 6.71 (br. s)</td>
<td>6.58 (br. s), 7.14 (br. s)</td>
<td>18</td>
<td></td>
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[a] Recorded at 400 MHz. [b] Recorded at 100 MHz.

Table 2 continued...
products to be reported from the plant endophytic fungus P. brasiliense.

Experimental Section

General: Optical rotations were measured with a Perkin–Elmer 241 polarimeter, and UV data were recorded with a Shimadzu Biocom-1601 spectrophotometer. CD spectra were recorded with a JASCO J-815 spectropolarimeter by using CHCl3 as the solvent. IR data were recorded using a Nicolet Magna-IR 750 spectrometer. 1H and 13C NMR spectroscopic data were acquired with Varian Mercury-400 and ~500 spectrometers using the solvent signals as references ([D6]acetone: δ = 2.05/29.8, 206.1 ppm). HMOC and HMBC experiments were optimized for 145.0 and 8.0 Hz, respectively. MS and HRMS (ESI) data were recorded with a Mariner ESI-TOF mass spectrometer.

Fungal Material: The culture of Paraconiothyrium brasiliense Verkley was isolated by one of the authors (L.G.) from branches of Acer truncatum Bunge on Dongling Mountain, Beijing, in March, 2005. The isolate was identified and assigned the accession number M3—Bunge on Dongling Mountain, Beijing, in March, 2005. The isolate was identified and assigned the accession number M3–Bunge on Dongling Mountain, Beijing, in March, 2005.

Full Paper

X-ray Crystallographic Analysis of 1: Upon crystallization from MeOH/H2O (20:1) using the vapor diffusion method, colorless crystals were obtained for 1 and a crystal (0.54 × 0.17 × 0.17 mm) was separated from the sample and mounted on a glass fiber, and data were collected by using a Rigaku R-AXIS Rapid IP diffractometer with graphite-monochromated Cu-Kα radiation (λ = 1.5418 Å) at 173(2) K. Crystal data: C13H18O6S, M = 293.31, space group monochlino, P21, unit cell dimensions: a = 10.0881(11) Å, b = 17.8498(17) Å, c = 19.85(2) Å, V = 1397.3(2) Å3, Z = 4, Dcalc = 1.394 mg·mL−1, μ = 0.874 mm−1, F(000) = 624. The structure was solved by direct methods by using SHELXL-97 and refined by 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 using the Siemens Area Detector Absorption Program (SADABS). The 16210 measurements yielded 4911 independent reflections after equivalent data had been averaged and Lorentz and polarization corrections applied. The final refinement gave R1 = 0.0386 and wR2 = 0.0882 [I > 2σ(I)].

Brasilamide B (2): Colorless oil. [α]D 20 = −8.0 (c = 0.1, MeOH). UV (MeOH): λ (log e, m−1·cm−1) = 215 (4.14) nm. CD (c = 6.1 × 10−3 M, CHCl3): [α]D = 238 (−0.78), 289 (−0.92) nm. IR (neat): ν = 3350 (br), 2931, 2927, 1707, 1670, 1594, 1452, 1377, 1056 cm−1. For 1H and 13C NMR spectroscopic data, see Table 2. HMBC (400 MHz, [D6]acetone, 25 °C): 1-H ↔ C-2, C-5, C-7, C-8, C-9, C-11; 3-H ↔ C-1, C-2, C-5, C-7, 6-H ↔ C-2, C-5, C-7, C-8, C-9; 7-H ↔ C-1, C-2, C-5, C-8, C-11; 8a-H ↔ C-1, C-2, C-3, C-6, C-7, C-9; 8b-H ↔ C-1, C-2, C-5, C-6, C-7, C-8, C-11; 9-H ↔ C-1, C-2, C-3, C-6, C-7, C-8, C-9; 11-H ↔ C-1, C-2, C-3, C-6, C-7, C-9; 12-H ↔ C-1, C-13; 13-H ↔ C-11, C-12, C-15, C-16; 16-H ↔ C-13, C-14, C-15; 6-OH ↔ C-5, C-6, C-9. NOESY (500 MHz, [D6]acetone, 25 °C): 1-H ↔ 10-H; 3-H ↔ 10-H, 11-H; 8a-H ↔ 9-H; 8b-H ↔ 11-H; 9-H ↔ 8a-H; 10-H ↔ 1-H, 3-H; 11-H ↔ 3-H, 8b-H. HRMS (ESI): calcd for C13H18O6Na2 [M + Na]+ 316.1155; found 316.1147.

Absolute Conuration of the Tertiary Alcohol in 2: According to the published procedure, a sample of 2 (0.5 mg) was dissolved in a dry solution of the stock [Rh2(OOCCH3)4] complex (0.8 mg) in CH3Cl (300 mL) and was subjected to CD measurements at a concentration of 1.7 mg·mL−1. The first CD spectrum was recorded immediately after mixing and its time evolution was monitored until stationary (ca. 10 min after mixing). The inherent CD was subtracted. The observed sign of the E band at around 350 nm in the induced CD spectrum was correlated to the absolute configuration of the C-5 tertiary alcohol moiety.

Brasilamide C (3): Colorless powder. [α]D 20 = +69 (c = 0.1, MeOH). UV (MeOH): λ (log e, m−1·cm−1) = 213 (4.17) nm. IR (neat): ν = 3350 (br), 2934, 2869, 1751, 1679, 1603, 1380, 1194, 1083 cm−1. For 1H and 13C NMR spectroscopic data, see Table 2. HMBC data (400 MHz, [D6]acetone, 25 °C): 2-H ↔ C-1, C-4, C-6, C-10; 3-H ↔ C-1, C-2, C-4, C-5, C-7; 4-H ↔ C-2, C-3, C-5, C-6, C-7, C-8, C-11; 5a-H ↔ C-1, C-4, C-6, C-7, 5b-H ↔ C-1, C-4, C-6, C-7, C-8, C-11; 6-H ↔ C-1, C-2, C-4, C-5, C-7, C-10, C-11; 7-H ↔ C-1, C-4, C-6, C-7, C-8, C-11; 10-H ↔ C-1, C-4, C-6, C-7, C-8, C-11; 11-H ↔ C-1, C-4, C-6, C-7, C-8, C-11; 12-H ↔ C-1, C-4, C-6, C-7, C-8, C-11; NOESY correlations (500 MHz, [D6]acetone, 25 °C): 2b-H ↔ 5a-

Brasilamides D (4): Colorless powder. [α]_{D}^{25} +63 (c = 0.1, MeOH). UV (MeOH): λ (logε, M⁻¹cm⁻¹) = 210 (4.15) nm. IR (neat): ν = 3347 (br.), 2929, 2879, 1736, 1669, 1604, 1369, 1243, 1038 cm⁻¹. For ¹H and ¹³C NMR spectroscopic data, see Table 2. HMBC (provided by Dr. Bin Yan) was added and incubated at 37 °C for 1 h. After 1H and 13C NMR spectra of brasilamides A were recorded. Colorless powder. λ_{UV} (MeOH): ε = 210 (4.15) nm. IR (neat): ν = 3347 (br.), 2929, 2879, 1736, 1669, 1604, 1369, 1243, 1038 cm⁻¹. For ¹H and ¹³C NMR spectroscopic data, see Table 2. HMBC (provided by Dr. Bin Yan) was added and incubated at 37 °C for 1 h. After 1H and 13C NMR spectra of brasilamides A were recorded.

Anti-HIV Assays: Anti-HIV assays included cytotoxicity and HIV-1 replication inhibition evaluations. Cells (3 × 10⁵/well) were seeded into a 96-well microtiter plate in the absence or presence of various concentrations of test compounds in triplicate and incubated at 37 °C in a humid atmosphere of 5% CO₂. After incubation for 4 d, cell viability was measured by the MTT method. The concentration that caused the reduction of viable cells by 50% (CC₅₀) was determined. In parallel with the MTT assay, a HIV-1 replication inhibition assay was determined by p24 antigen capture ELISA. C8166 cells were exposed to HIV-1 at 37 °C for 1.5 h, washed with PBS (phosphate-buffered saline) to remove free viruses and seeded into a 96-well microtiter plate at 3 × 10⁴ cells per well in the absence or presence of test compounds (indinavir sulfate was used as positive control). After 4 d, the supernatant was collected and inactivated with 0.5% Triton X-100. The supernatant was diluted in the absence or presence of test compounds (indinavir sulfate was used as positive control). After 4 d, the supernatant was collected and inactivated with 0.5% Triton X-100. The supernatant was diluted.

Supporting Information (see footnote on the first page of this article): ¹H and ¹³C NMR spectra of brasilamides A–D (1–4).

Acknowledgments

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[27] CCDC-765974 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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Paraconiothyrium brasiliense produced four new tricyclic sesquiterpenoids named brasilamides A–D (1–4); compounds 1 and 2 possess an unprecedented 4-oxatricyclo[3.3.1.0³⁷]nonane skeleton. Compounds 2–4 showed modest inhibitory effects on HIV-1 replication in C8166 cells.

Keywords: Antiviral agents / Biological activity / Structure elucidation / Natural products / Configuration determination