

# Penibругuieramine A, a Novel Pyrrolizidine Alkaloid from the Endophytic Fungus *Penicillium* sp. GD6 Associated with Chinese Mangrove *Bruguiera gymnorrhiza*

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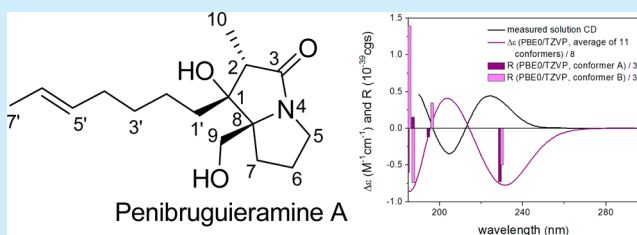
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## S Supporting Information

**ABSTRACT:** A novel pyrrolizidine alkaloid, penibругuieramine A (1), characterized by an unprecedented 1-alkenyl-2-methyl-8-hydroxymethylpyrrolizidin-3-one skeleton, was isolated from the endophytic fungus *Penicillium* sp. GD6, associated with the Chinese mangrove *Bruguiera gymnorrhiza*. The absolute configuration of penibругuieramine A (1) was established by TDDFT ECD calculations of the vacuum and solution conformers, exploiting the transitions of the lactam chromophore. A plausible pathway for its biosynthesis has been proposed.



Marine microorganisms are now recognized as a source of structurally unique and biopharmacologically interesting natural products, and they have been receiving considerable attention.<sup>1</sup> Among them, fungal strains from marine plants constitute a hotspot in the recent research on endophytic fungi; in particular, fungi from mangrove habitats have shown great potential in the production of biologically active secondary metabolites.<sup>2</sup>

The genus *Penicillium* is one of the largest and most intensively investigated mangrove endophytic fungal genera.<sup>3</sup> A wide array of bioactive secondary metabolites from mangrove endophytic *Penicillium* species have been characterized, including meroterpenoid and diphenyl ether derivatives,<sup>4</sup> cytotoxic polyphenols,<sup>5</sup> azaphilones, and *p*-terphenyls.<sup>6</sup> In the course of our continuing efforts aimed at finding new bioactive substances from marine microorganisms,<sup>7</sup> a fungal strain *Penicillium* sp. GD6, recently isolated from the Chinese mangrove *Bruguiera gymnorrhiza* collected off the coasts of Zhanjiang, China, was selected for further investigation since its crude EtOAc extract showed potent antibacterial activity against *Staphylococcus aureus* with an MIC value of 6.4 μg/mL. A chemical investigation of the whole culture of *Penicillium* sp. GD6 has resulted in the isolation of a novel pyrrolizidine alkaloid, named penibругuieramine A (1), characterized by an unprecedented 1-alkenyl-2-methyl-8-hydroxymethyl pyrrolizidin-3-one skeleton. Herein, we describe the isolation, structure elucidation, and postulated biogenetic origin of this novel compound.

The whole culture of *Penicillium* sp., strain GD6 (30L) was extracted exhaustively with EtOAc at room temperature. Then, the extract was subjected to repeated chromatography over MCI gel, silica gel, and C-18 reversed-phase silica gel to yield penibругuieramine A (1) (Supporting Information). In addition, three known compounds, namely scalusamide A (2),<sup>8</sup> meleagrins (3),<sup>9</sup> and roquefortine F (4),<sup>10</sup> were also isolated during the purification process of 1 (Figure 1).

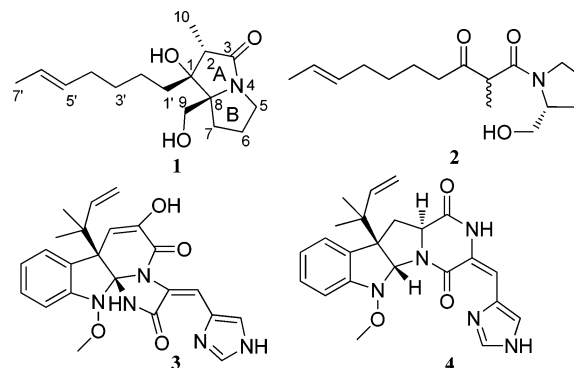


Figure 1. Structures of compounds 1–4.

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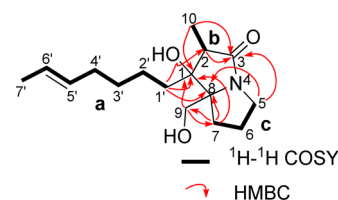
Penibругuieramine A (**1**),<sup>11</sup> was isolated as a colorless gum. Its molecular formula was deduced to be C<sub>16</sub>H<sub>27</sub>NO<sub>3</sub> by HR-ESIMS 304.1883 [M + Na]<sup>+</sup> (calcd 304.1889), suggesting the presence of four degrees of unsaturation. The IR spectrum showed a broad absorption at 3425 cm<sup>-1</sup>, suggesting the presence of hydroxyl group(s), and an absorption at  $\nu_{\max}$  1680 cm<sup>-1</sup>, in agreement with the presence of a five-membered lactam carbonyl. The <sup>13</sup>C NMR spectrum of **1** (Table 1),

**Table 1.** <sup>1</sup>H (500 MHz) and <sup>13</sup>C NMR (125 MHz) Data for **1** in CDCl<sub>3</sub>

no.	$\delta_{\text{H}}$ , mult. (J in Hz)	$\delta_{\text{C}}$
1		81.6, qC
2	2.98, q (7.2)	49.1, CH
3		177.4, qC
5 <sub>ax</sub>	2.94, m	42.8, CH <sub>2</sub>
5 <sub>eq</sub>	3.78, m	
6 <sub>ax</sub>	1.86, ddd (12.2, 12.9, 6.3)	26.5, CH <sub>2</sub>
6 <sub>eq</sub>	1.95, m	
7 <sub>ax</sub>	2.28, ddd (13.5, 11.0, 6.3)	27.2, CH <sub>2</sub>
7 <sub>eq</sub>	1.47, m	
8		75.8, qC
9 <sub>a</sub>	3.54, d (11.9)	65.8, CH <sub>2</sub>
9 <sub>b</sub>	3.71, d (11.9)	
10	1.06, d (7.2)	7.1, CH <sub>3</sub>
1' <sub>a</sub>	1.61, dd (14.5, 4.5)	34.2, CH <sub>2</sub>
1' <sub>b</sub>	1.72, m	
2' <sub>a</sub>	1.54, m	23.2, CH <sub>2</sub>
2' <sub>b</sub>	1.24, m	
3' <sub>a</sub>	1.38, m	30.0, CH <sub>2</sub>
3' <sub>b</sub>	1.26, m	
4'	2.00, m	32.3, CH <sub>2</sub>
5'	5.41, m	130.9, CH
6'	5.41, m	125.2, CH
7'	1.65, d (4.9)	17.9, CH <sub>3</sub>

analyzed by means of the 2D HSQC experiments, evidenced resonances of two methyls, eight sp<sup>3</sup> methylenes (one oxygenated), two sp<sup>2</sup> methines, one sp<sup>3</sup> methine, two sp<sup>3</sup> quaternary carbons, and one amide carbonyl. The presence of one disubstituted double bond was easily recognized by its <sup>1</sup>H and <sup>13</sup>C NMR resonances [ $\delta_{\text{H}}$  5.41, m, 2H, H-5' and H-6';  $\delta_{\text{C}}$  130.9 (C-5') and 125.2 (C-6')]. Resonances arising from two methyls, including one vinyl methyl ( $\delta$  1.65, d,  $J$  = 4.9 Hz) and one secondary methyl ( $\delta$  1.06, d,  $J$  = 7.2 Hz), were also observed in the <sup>1</sup>H NMR spectrum of **1** (Table 1). Moreover, the appearance of a pair of AB-type proton signals at  $\delta$  3.71 (1H, d,  $J$  = 11.9 Hz) and 3.54 (1H, d,  $J$  = 11.9 Hz), coupled in the HSQC spectrum with the resonance at  $\delta$  65.8, clearly suggested the presence of an uncoupled oxymethylene group. Since the carbon–carbon double bond and the amide carbonyl accounted for only two of the required four sites of unsaturation, penibругuieramine A (**1**) must be bicyclic.

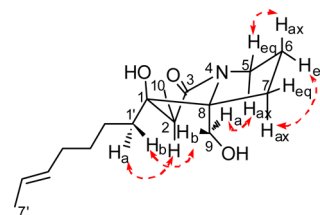
Analysis of the <sup>1</sup>H–<sup>1</sup>H COSY spectrum of **1**, aided by HSQC experiment, revealed three proton connectivities shown bolded in Figure 2, and identified as a 2-hepten-7-yl moiety (a), –CHCH<sub>3</sub> (b), and –CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>– (c) fragments. These subunits, the amide carbonyl, two quaternary carbons ( $\delta_{\text{C}}$  75.8;  $\delta_{\text{C}}$  81.6), and the oxygenated methylene ( $\delta_{\text{C}}$  65.8), were connected by careful interpretation of the well-resolved HMBC spectrum. Particularly diagnostic were the HMBC cross peaks of H-7 with C-8, C-1, and C-9, of H<sub>2</sub>-1' with C-1, C-2, and C-8,



**Figure 2.** Key HMBC and <sup>1</sup>H–<sup>1</sup>H COSY correlations of penibругuieramine A (**1**).

and of H<sub>3</sub>–10 with C-1 and the carbonyl C-3. These correlations allowed the location of the alkenyl chain at the oxygenated quaternary C-1 and linkage of this latter carbon to the methylene-bearing C-8, which, in turn, must be connected to the fragment c via C-7. The presence of an amide group and the still “loose end” of a quaternary carbon at  $\delta_{\text{C}}$  75.8 (C-8) were rationalized with the aid of the HMBC correlations of H<sub>2</sub>-5 with C-3 and C-8, indicating that C-5 and C-8 must be both attached to the amide nitrogen. The consequent formation of a pyrrolizidine ring system is in perfect agreement with the degrees of unsaturation. Thus, all the NMR data of **1** were unambiguously assigned in accordance with the planar structure of **1** as reported in Table 1.

The relative configuration of stereogenic carbons around the pyrrolizidine core was established by detailed analysis of the ROESY spectrum of **1**. As shown in Figure 3, the ROESY

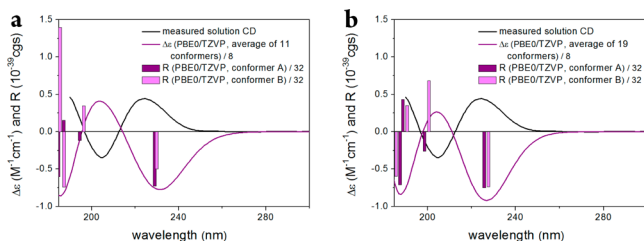


**Figure 3.** Key ROESY correlations for penibругuieramine A (**1**) (relative configuration shown).

correlations of H<sub>a</sub>-9/H<sub>ax</sub>-5, H<sub>eq</sub>-6/H<sub>ax</sub>-7, H<sub>b</sub>-9/H<sub>b</sub>-1', and H<sub>a</sub>-1'/H-2 indicated that H<sub>2</sub>-9, H<sub>2</sub>-1', H-2, H<sub>ax</sub>-5, H<sub>eq</sub>-6, and H<sub>ax</sub>-7 were all cofacial, arbitrarily assigned as  $\alpha$ -oriented. The opposite ( $\beta$ ) orientation of H<sub>ax</sub>-6 and H<sub>eq</sub>-5 was established by the NOE cross-peak of H<sub>ax</sub>-6/H<sub>eq</sub>-5. Thus, the relative configuration of **2** was determined as (1S\*,2R\*,8R\*). The *trans* geometry of the  $\Delta^{5'(6')}$  double bond was deduced by the <sup>13</sup>C NMR chemical shift of C-7', resonating at  $\delta_{\text{C}}$  17.9 (<20 ppm).<sup>12</sup>

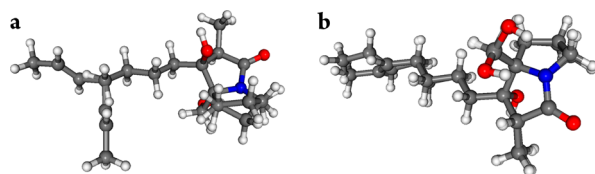
The absolute configuration of **1** was determined by comparison of its experimental and Time-Dependent Density Functional Theory (TDDFT) calculated electronic circular dichroism (ECD) spectra. This computational approach has proved to be a powerful and reliable method in determining the absolute configuration of natural products, even with flexible skeletons.<sup>13</sup> The ECD spectrum (CH<sub>3</sub>CN) of **1** showed weak transitions of the lactam chromophore with a tertiary nitrogen: a positive  $n$ – $\pi^*$  Cotton effect (CE) at 223 nm ( $\Delta\epsilon$  = 0.45), a negative  $\pi$ – $\pi^*$  one at 204 nm ( $\Delta\epsilon$  = –0.39), and a positive  $\pi$ – $\pi^*$  CE at 192 ( $\Delta\epsilon$  = 0.28). The initial Merck Molecular Force Field (MMFF) conformational analysis of (1S,2R,8R)-**1** afforded 1113 conformers, which were reclustered excluding the different orientations of the C-1 alkenyl side chain. The resulting 31 geometries were reoptimized at the B3LYP/6-31G(d) level in vacuo and B3LYP/TZVP level in CH<sub>3</sub>CN

(polarizable continuum model). The reoptimization in vacuo produced 11 conformers above 1% population (Supporting Information, Figure S1), for which ECD spectra were calculated with the TZVP basis set and three different functionals (B3LYP, BH&HLYP, PBE0). With all three methods, the Boltzmann-averaged ECD spectra of (1*S*,2*R*,8*R*)-**1** appeared as a mirror image curve compared to the experimental one, thus suggesting a (1*R*,2*S*,8*S*) absolute configuration (Figure 4a).



**Figure 4.** Experimental ECD spectrum of penibruquiamine A (**1**) in acetonitrile compared with the Boltzmann-weighted PBE0/TZVP spectra calculated for (1*S*,2*R*,8*R*)-**1**: (a) in vacuo; (b) with PCM solvent model for CH<sub>3</sub>CN. Bars show the rotatory strength of the two lowest-energy conformers; conformer A (49.0% and 22.3% population in vacuo and with PCM model, respectively) and conformer B (10.6% and 12.2% population in vacuo and with PCM model, respectively).

Compared to the experimental ECD, the lowest-energy conformer A (49.0% population) showed oppositely signed CEs for the 223 and 192 nm transitions, while it has the same negative sign for the 204 nm transition. In contrast, conformer B (10.6% population) and the higher-energy conformers gave mirror image CEs of the experimental curve for all the transitions. The 223 and 204 nm CEs derive from a single transition, while the 192 nm one is the sum of two overlapping oppositely signed transitions. The overlapped geometries of conformers A and B (Figure 5a) revealed that they differ in the conformation of ring B and the orientation of the 2-hepten-7-yl side chain.



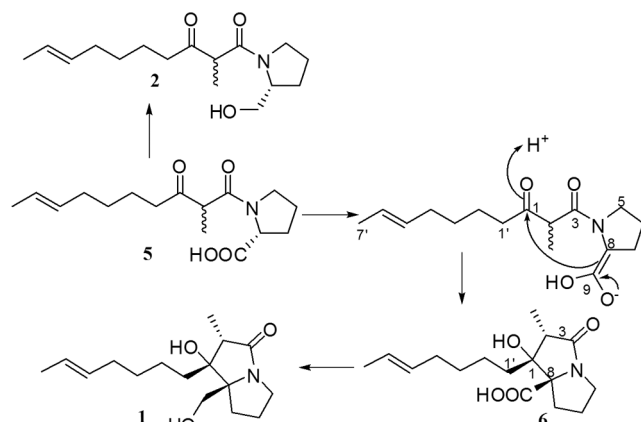
**Figure 5.** (a) Overlapped geometries of conformer A (49.0%) and B (10.6%) obtained by in vacuo B3LYP/6-31G reoptimization. (b) Overlapped geometries of conformer A (22.3%) and B (12.2%) obtained by B3LYP/TZVP reoptimization with PCM solvent model for CH<sub>3</sub>CN.

The B3LYP/TZVP reoptimization with the PCM solvent model for CH<sub>3</sub>CN produced 19 conformers (Supporting Information, Figure S2), and it identified the same lowest-energy conformer as the in vacuo optimization, although less populated (22.3%). Conformer B (12.2%) (Figure 5b) was different from the parallel “in vacuo” conformer, but their ECD curves were very similar. In solution, the higher-energy conformers were also responsible for the positive sign of the 204 nm band in the computed average ECD spectra. The close similarity of the Boltzmann-weighted ECD spectra calculated with different methods for the in vacuo and solvent model

conformers (Figure 4b) confirms the unambiguous assignment of the absolute configuration of **1** as (1*R*,2*S*,8*S*).

Penibruquiamine A (**1**) is a novel pyrrolizidine alkaloid characterized by the unprecedented 1-hydroxyl-2-methyl pyrrolizidin-3-one skeleton bearing an unbranched alkenyl chain at C-1 and a hydroxymethyl group at C-8. A proposed pathway for the biosynthesis of **1** is outlined in Scheme 1. A likely

**Scheme 1.** Proposed Biosynthetic Pathway for **1**



biogenetic precursor is the proline-pentaketide amide **5**, already proposed to act as precursors of the co-occurring alkaloid scalusamide A (**2**)<sup>8</sup> and of another related *Penicillium* alkaloid, perinadine A.<sup>14</sup> A Claisen-type reaction between C-8 and the ketone carbonyl at C-1 could give the acid **6**, the direct precursor of the target molecule **1**.

Although the crude extract showed promising antibacterial activity as mentioned before, compounds **2** and **3** proved to be inactive against the methicillin-sensitive *S. aureus* Newman strain, while compound **3** showed potent cytotoxic activity against two tumor cell lines, HL60 and A549, with IC<sub>50</sub> values of 9.7 and 8.3 μM, respectively. Due to the scarcity of material, the antibacterial and cytotoxic activities of **1** and **4** were not evaluated.

Many pyrrolizidine alkaloids have been isolated from higher plants, which produce them as a defense mechanism against the attack of herbivores. A single example of a related polyhydroxylated pyrrolizidine alkaloid has been reported from the fungus *Pochonia suchlasporia* var. *suchlasporia* TAMA 87.<sup>15</sup> Typically, these compounds bear a 1-hydroxymethyl-7-hydroxy-pyrrolizidine skeleton and structural variability is generally a result of nitrogen oxidation/methylation, oxygen acylation, and carbon hydroxylation.<sup>16</sup> Thus, penibruquiamine A (**1**) not only qualifies as the first pyrrolizidine alkaloid obtained from an endophytic *Penicillium* fungus but it also bears an unprecedented alkylation/functionalization pattern around the pyrrolizidine system. Accordingly, also the biosynthetic pathway generating the pyrrolizidine system of **1** (as proposed in Scheme 1) is likely to be completely alternative compared to that demonstrated for pyrrolizidine-based alkaloids found in higher plants.<sup>16</sup>

Further studies should be conducted to understand the effective biological role of penibruquiamine A (**1**) in the life cycle and ecology of the fungus *Penicillium* sp. and its host plant, as well as to explore the metabolites responsible for the antibacterial activity.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

Experimental procedures, full NMR spectra for compounds 1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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- (11) Penibругuieramine A (1): colorless gum;  $[\alpha]_D^{22}$   $-22$  (c 0.05, CHCl<sub>3</sub>); IR (KBr)  $\nu_{\max}$  3425 (broad), 2931, 1680, 1640, 968, 578 cm<sup>-1</sup>; UV (CH<sub>3</sub>CN)  $\lambda_{\max}$  (log  $\epsilon$ ): 202 (0.46) nm; ECD (CH<sub>3</sub>CN, c = 1.07 × 10<sup>-3</sup>)  $\Delta\epsilon$  (nm) +0.45 (223), -0.39 (204), +0.28 (192); <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 1; ESIMS  $m/z$  282.2 [M + H]<sup>+</sup>; HRESIMS  $m/z$  304.1883 ([M + Na]<sup>+</sup>, calcd for C<sub>16</sub>H<sub>27</sub>NO<sub>3</sub>Na 304.1889).
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