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

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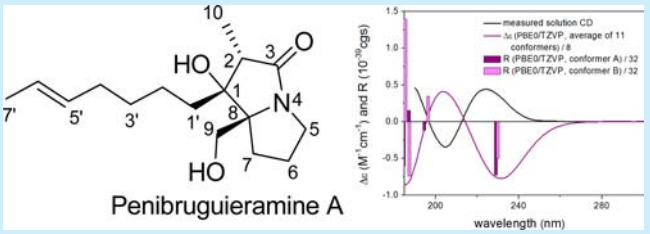
Supporting Information

ABSTRACT: A novel pyrrolizidine alkaloid, penibruguieramine 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 penibruguieramine 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.[1] 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.[2]

The genus *Penicillium* is one of the largest and most intensively investigated mangrove endophytic fungal genera.[3] A wide array of bioactive secondary metabolites from mangrove endophytic *Penicillium* species have been characterized, including meroterpenoid and diphenyl ether derivatives,[4] cytotoxic polyphenols,[5] azaphilones, and *p*-terphenyls.[6] In the course of our continuing efforts aimed at finding new bioactive substances from marine microorganisms,[7] 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 penibruguieramine 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 penibruguieramine A (**1**) (Supporting Information). In addition, three known compounds, namely scalusamide A (**2**),[8] meleagrin (**3**),[9] and roquefortine F (**4**),[10] were also isolated during the purification process of **1** (Figure 1).

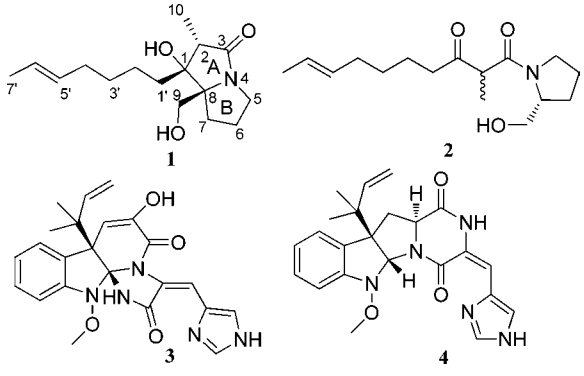


Figure 1. Structures of compounds **1**−**4**.

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Penibruguieramine A (**1**),[11] was isolated as a colorless gum. Its molecular formula was deduced to be $C_{16}H_{27}NO_3$ by HR-ESIMS 304.1883 $[M + Na]^+$ (cald 304.1889), suggesting the presence of four degrees of unsaturation. The IR spectrum showed a broad absorption at 3425 cm^{-1}, suggesting the presence of hydroxyl group(s), and an absorption at υ_{max} 1680 cm^{-1}, in agreement with the presence of a five-membered lactam carbonyl. The ^{13}C NMR spectrum of **1** (Table 1),

Table 1. ^1H (500 MHz) and ^{13}C NMR (125 MHz) Data for 1 in $CDCl_3$

no.	δ_H, mult. (J in Hz)	δ_C
1		81.6, qC
2	2.98, q (7.2)	49.1, CH
3		177.4, qC
5ax	2.94, m	42.8, CH_2
5eq	3.78, m	
6ax	1.86, ddd (12.2, 12.9, 6.3)	26.5, CH_2
6eq	1.95, m	
7ax	2.28, ddd (13.5, 11.0, 6.3)	27.2, CH_2
7eq	1.47, m	
8		75.8, qC
9a	3.54, d (11.9)	65.8, CH_2
9b	3.71, d (11.9)	
10	1.06, d (7.2)	7.1, CH_3
1′a	1.61, dd (14.5, 4.5)	34.2, CH_2
1′b	1.72, m	
2′a	1.54, m	23.2, CH_2
2′b	1.24, m	
3′a	1.38, m	30.0, CH_2
3′b	1.26, m	
4′	2.00, m	32.3, CH_2
5′	5.41, m	130.9, CH
6′	5.41, m	125.2, CH
7′	1.65, d (4.9)	17.9, CH_3

analyzed by means of the 2D HSQC experiments, evidenced resonances of two methyls, eight sp^3 methylenes (one oxygenated), two sp^2 methines, one sp^3 methine, two sp^3 quaternary carbons, and one amide carbonyl. The presence of one disubstituted double bond was easily recognized by its ^1H and ^{13}C NMR resonances [δ_H 5.41, m, 2H, H-5′ and H-6′; δ_C 130.9 (C-5′) and 125.2 (C-6′)]. Resonances arising from two methyls, including one vinyl methyl (δ 1.65, d, J = 4.9 Hz) and one secondary methyl (δ 1.06, d, J = 7.2 Hz), were also observed in the ^1H NMR spectrum of **1** (Table 1). Moreover, the appearance of a pair of AB-type proton signals at δ 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 δ 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, penibruguieramine A (**1**) must be bicyclic.

Analysis of the $^1H-^1H$ 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**), $-CHCH_3$ (**b**), and $-CH_2CH_2CH_2-$ (**c**) fragments. These subunits, the amide carbonyl, two quaternary carbons (δ_C 75.8; δ_C 81.6), and the oxygenated methylene (δ_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_2-1′ with C-1, C-2, and C-8,

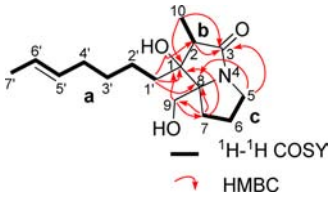

Figure 2. Key HMBC and $^1H-^1H$ COSY correlations of penibruguieramine A (**1**).

and of H_3-10 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 δ_C 75.8 (C-8) were rationalized with the aid of the HMBC correlations of H_2-5 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

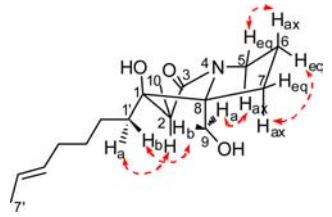

Figure 3. Key ROESY correlations for penibruguieramine A (**1**) (relative configuration shown).

correlations of H_a-9/H_{ax}-5, H_{eq}-6/H_{ax}-7, H_b-9/H_b-1′, and H_a-1′/H-2 indicated that H_2-9, H_2-1′, H-2, H_{ax}-5, H_{eq}-6, and H_{ax}-7 were all cofacial, arbitrarily assigned as α-oriented. The opposite (β) orientation of H_{ax}-6 and H_{eq}-5 was established by the NOE cross-peak of H_{ax}-6/H_{eq}-5. Thus, the relative configuration of **2** was determined as (1*S**,2*R**,8*R**). The *trans* geometry of the $\Delta^{5'(6')}$ double bond was deduced by the ^{13}C NMR chemical shift of C-7′, resonating at δ_C 17.9 (<20 ppm).[12]

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.[13] The ECD spectrum (CH_3CN) of **1** showed weak transitions of the lactam chromophore with a tertiary nitrogen: a positive n−π* Cotton effect (CE) at 223 nm ($\Delta\varepsilon$ = 0.45), a negative $\pi-\pi$* one at 204 nm ($\Delta\varepsilon$ = −0.39), and a positive $\pi-\pi$* CE at 192 ($\Delta\varepsilon$ = 0.28). The initial Merck Molecular Force Field (MMFF) conformational analysis of (1*S*,2*R*,8*R*)-**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_3CN

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(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).

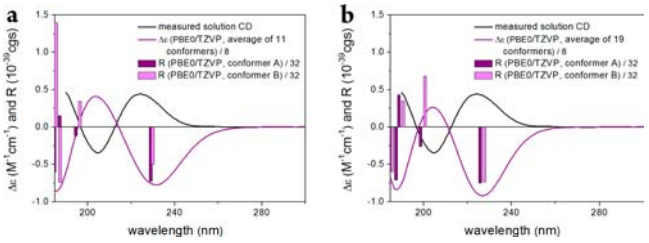


Figure 4. Experimental ECD spectrum of penibruguieramine A (**1**) in acetonitrile compared with the Boltzmann-weighted PBE0/TZVP spectra calculated for (1*S*,2*R*,8*R*)-**1**: (a) in vacuo; (b) with PCM solvent model for CH_3CN. Bars show the rotatory strength of the two lowest-energy conformers; conformer 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.

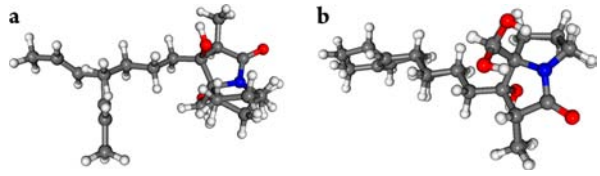
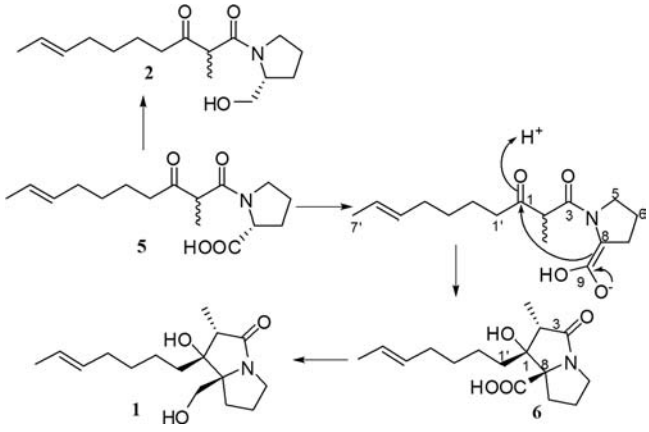


Figure 5. (a) Overlapped geometries of conformer A (49.0%) and B (10.6%) obtained by in vacuo B3LYP/6-31G reoptimization. (b) Overlapped geometries of conformer A (22.3%) and B (12.2%) obtained by B3LYP/TZVP reoptimization with PCM solvent model for CH_3CN.

The B3LYP/TZVP reoptimization with the PCM solvent model for CH_3CN 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*).

Penibruguieramine 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**)[8] and of another related *Penicillium* alkaloid, perinadine A.[14] 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_{50} 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.[15] 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.[16] Thus, penibruguieramine 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.[16]

Further studies should be conducted to understand the effective biological role of penibruguieramine 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.

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■ 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.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

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(11) Penibruguieramine A (**1**): colorless gum; $[\alpha]_D^{22}$ −22 (*c* 0.05, $CHCl_3$); IR (KBr) ν_{max} 3425 (broad), 2931, 1680, 1640, 968, 578 cm^{-1}; UV (CH_3CN) λ_{max} (log ε): 202 (0.46) nm; ECD (CH_3CN, $c = 1.07 \times 10^{-3}$) $\Delta\varepsilon$ (nm) +0.45 (223), −0.39 (204), +0.28 (192); ^1H and ^{13}C NMR data, see Table 1; ESIMS *m*/*z* 282.2 $[M + H]^+$; HRESIMS *m*/*z* 304.1883 ($[M + Na]^+$, calcd for $C_{16}H_{27}NO_3Na$ 304.1889).

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