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Penicibilaenes A and B, Sesquiterpenes with a Tricyclo[6.3.1.0^{1,5}]dodecane Skeleton from the Marine Isolate of *Penicillium bilaiae* MA-267

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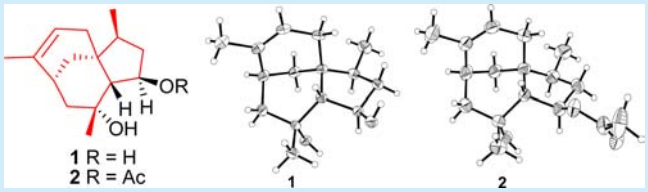
Supporting Information

ABSTRACT: Penicibilaenes A (**1**) and B (**2**), two sesquiterpenes possessing a tricyclo[6.3.1.0^{1,5}]dodecane skeleton, were characterized from *Penicillium bilaiae* MA-267, a fungus obtained from the rhizospheric soil of the mangrove plant *Lumnitzera racemosa*. The lack of some key COSY and NOESY correlations made the structure elucidation of compound **1** difficult, which was solved by a X-ray crystallographic study. Compounds **1** and **2** exhibited selective activity against the plant pathogenic fungus *Colletotrichum gloeosporioides* (MIC = 1.0 and 0.125 μg/mL, respectively).



The marine ecosystem is a rich reservoir for structurally unique and biologically active secondary metabolites.[1,2] In the course of our ongoing research on marine-derived fungi,[3−6] we recently focused our attention on an isolate of *Penicillium bilaiae* MA-267 that was obtained from the rhizospheric soil of the marine mangrove plant *Lumnitzera racemosa*. *P. bilaiae* is a species of native soil fungus that can be used as a plant-growth-promoting micro-organism, but its chemical constituents have rarely been studied. To our knowledge, only one paper describing the isolation of an acetylenic nematicidal compound (penipratynolene) has been reported.[7] In our preliminary bioassay, the culture extract of *P. bilaiae* MA-267 exhibited inhibitory activity against *Colletotrichum gloeosporioides*, a plant pathogenic fungus that causes anthracnose on many fruit and vegetable species such as mango, papaya, tomato tree fruit, quince, and apple.[8,9] Chemical work on the active fraction of *P. bilaiae* MA-267 afforded two new sesquiterpenes, penicibilaenes A and B (**1** and **2**, Figure 1), each of which contains a tricyclo[6.3.1.0^{1,5}]dodecane framework. This paper describes the isolation, structure determination, stereochemical assignment, and antimicrobial activity as well as the plausible biogenetic pathway of compounds **1** and **2**.

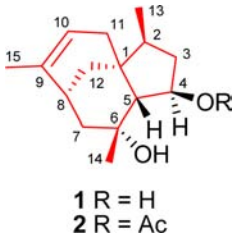


Figure 1. Chemical structures of compounds **1** and **2**.

The fungus *P. bilaiae* MA-267, which was identified by analysis of its ITS (internal transcript spacer) sequence (Genbank accession no. KP096311), was cultivated in PDB (potato dextrose broth) medium, and the culture was exhaustively extracted with EtOAc to afford an extract which was further purified by repeated column chromatography on silica gel, Sephadex LH-20, and Lobar LiChroprep RP-18, to yield two new sesquiterpenes (**1** and **2**).

Penicibilaene A (**1**),[10] initially obtained as a colorless amorphous powder, was determined to have the molecular formula $C_{15}H_{24}O_2$ on the basis of positive HR-ESI-MS (*m*/*z* 259.1668 [M + Na]$^+$, calcd 259.1669). Inspection of ^{13}C NMR and DEPT (Table 1) spectra of **1** revealed the presence of three methyls, four aliphatic methylenes, five methines (with one olefinic and one oxygenated), and three nonprotonated carbons (with one olefinic and one bonded to oxygen). The molecular formula requires four degrees of unsaturation, but only two olefinic carbons resonating at δ_C 120.7 (CH, C-10) and 140.9 (C, C-9) were detected, indicating the tricyclic nature of compound **1**.

The ^{1}H NMR spectrum for **1** showed well-dispersed signals over a wide field range (Table 1 and Supporting Information), and aided by the HSQC experiment, these signals were revealed to represent three methyls (one doublet and two singlets), four aliphatic methylenes, and five methines (with one olefinic and one bonded to oxygen), which agreed with the ^{13}C NMR data. In addition, the ^{1}H NMR and HSQC spectra also revealed

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Table 1. NMR Data for Compounds 1 and 2

	1 (acquired in acetone-d_6)		2 (acquired in $CDCl_3$)	
no.	δ_C[a]	δ_H (mult, J in Hz)[b]	δ_C[a]	δ_H (mult, J in Hz)[b]
1	42.58		41.5	
2	42.62	1.69 (m)	42.1	1.80 (m)
3α	42.5	2.07 (m)	38.6	2.28 (ddd, 12.5, 7.1, 6.6)
3β		1.38 (dt, 11.7, 8.6)		1.34 (dt, 12.5, 8.7)
4	73.4	4.45 (m)	75.8	5.33 (ddd, 8.7, 6.6, 6.0)
5	61.5	1.46 (d, 6.2)	57.1	1.70 (d, 6.0)
6	71.3		71.3	
7α	33.3	1.86 (dd, 12.0, 4.8)	32.2	1.78 (dd, 11.9, 6.2)
7β		1.30 (dd, 12.0, 3.9)		1.41 (dd, 11.9, 2.5)
8	36.1	2.15 (dd, 4.8, 3.9)	34.7	2.18 (dd, 6.2, 2.5)
9	140.9		140.5	
10	120.7	5.23 (dd, 3.1, 1.5)	119.7	5.24 (d, 4.2)
11α	35.4	2.01 (d, 16.3)	34.4	2.02 (d, 16.0)
11β		1.74 (m)		1.70 (m)
12α	42.4	1.90 (dd, 14.2, 5.6)	41.9	1.75 (dd, 14.5, 6.3)
12β		1.50 (d, 14.2)		1.50 (d, 14.5)
13	15.0	0.89 (d, 7.1)	14.1	0.88 (d, 6.9)
14	31.4	1.26 (s)	30.5	1.13 (s)
15	22.2	1.63 (br s)	22.1	1.63 (m)
1′			171.1	
2′			21.6	1.97 (s)
4-OH		3.40 (d, 5.2)		
6-OH		3.20 (s)		

[a]Measured at 125 MHz, and multiplicities were determined by DEPT and HSQC experiments. [b]Measured at 500 MHz.

signals for two exchangeable protons that must correspond to hydroxyl groups given the absence of nitrogen and carbonyl carbons in **1**. The multiplicities of these exchangeable proton signals, one singlet at δ_H 3.20 and one doublet at δ_H 3.40, indicated their attachments to a nonprotonated carbon (C-6) and a methine carbon (C-4), respectively.

Detailed interpretation of the COSY and HSQC spectra of **1** resulted in the elucidation of three discrete proton spin-coupling systems corresponding to a $-CH_2-CH-$ unit (I, C-7 to C-8), a $CH_3-CH-CH_2-CH(OH)-CH-$ moiety (II, C-13 and C-2 through C-5), and a $=CH-CH_2-$ residue (III, C-10 to C-11) (Figure 2). HMBC correlations from H-7 to C-5 and

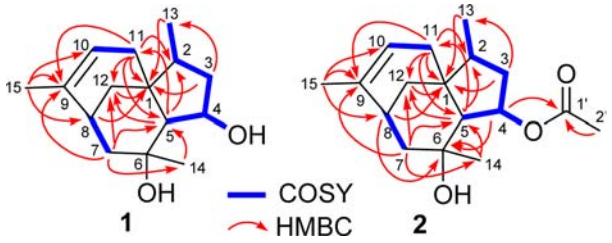

Figure 2. Selected 2D NMR correlations of **1** and **2**.

C-14 as well as from H_3-14 to C-5 connected the substructures I and II from C-7 to C-5 via the nonprotonated carbon C-6, while correlations from H-5 to C-2, C-11, and C-12, from H-3 and H_3-13 to C-1, from H-11 to C-1, C-2, C-5, and C-12, and from H-12 to C-1 suggested that C-2, C-5, C-11, and C-12 anchored to C-1, which proved the connection of units II and III from C-2 and C-5 to C-11 via C-1 and, therefore, led to the construction of the cyclopentane ring for **1** (Figure 2). Further HMBC correlations from H-7 and H-11 to C-9 and from H_3-15 to C-8, C-9, and C-10 verified the linkage of units I and III via nonprotonated olefinic carbon C-9. Finally, the connection of C-1 and C-8 through C-12, which allowed construction of the cyclohexane and cyclohexene rings to match the required degrees of unsaturation, was demonstrated by observation of the HMBC cross-peak from H-8 to C-1. However, the COSY correlation among H-8 and H_2-12 was not observed, making the connection of C-8 to C-12 not confidently assigned.

The relative configuration of penicibilaene A (**1**) was proposed by analysis of NOESY data (Figure 3). The NOE

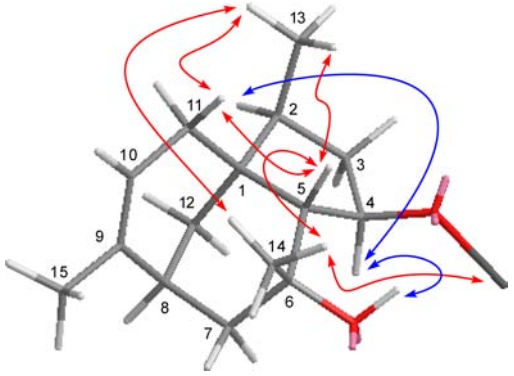

Figure 3. Key NOESY correlations observed for penicibilaene A (**1**).

correlations from H-5 to H-11, H-13, and H-14 and from H-14 to OH-4 indicated the cofacial orientation of these groups, while NOE cross-peaks from H-4 to H-2 and OH-6 placed these groups on the opposite face. Unfortunately, the relative configuration at C-8 could not be assigned since no diagnostic NOE correlation was observed in the NOESY spectrum.

The lack of some key COSY and NOESY correlations that could be used to fully elucidate the structure and relative configuration of **1** forced us to concentrate efforts toward a crystallographic study. Fortunately, X-ray quality crystals were cultivated upon slow evaporation of the solvents (MeOH–$CHCl_3$, 3:2) by keeping the sample in a refrigerator for 5 weeks.[10] The X-ray data not only confirmed the structure and relative configuration of **1** (Figure 4)[11] but also revealed that H-8 was nearly perpendicular to H_2-12, which caused no COSY correlation among them. The final refinement on the Cu Kα data resulted in a Flack parameter of 0.0(3), allowing an unequivocal assignment of the absolute configurations to be 1*R*, 2*S*, 4*R*, 5*R*, 6*R*, and 8*R*. Based on the above evidence, the structure of compound **1** was elucidated. This compound possesses a 2,6,9-trimethyltricyclo[6.3.1.$0^{1,5}$]dodecane skeleton, and to our knowledge, this skeleton only appears as a terpene core of hispidospermidin, a unique and more complex tetracyclic alkaloid characterized from *Chaetosphaeronema hispidulum* (Cda) Moesz NR 7127 by Ohtsuka and co-workers in 1994.[12] Based on the above discussion, the skeleton was tentatively named penicibilaene, and a trivial name penicibilaene A was assigned to compound **1**.

The obvious difference in the ^{1}H NMR spectra between compounds **1** and **2** was the appearance of an additional methyl singlet at δ_H 1.97 (H-1′), associated with the acetyl group, and downfield shift in the H-4 signal from δ_H 4.45 in **1** to δ_H 5.33 in **2**, as well as in the proton signals surrounding C-4. Two additional ^{13}C signals were observed at δ_C 171.1 (C, C-1′) and 21.6 (CH_3, C-2′) in the ^{13}C NMR spectrum for **2**, establishing the presence of an acetyl group in **2**. The observed HMBC

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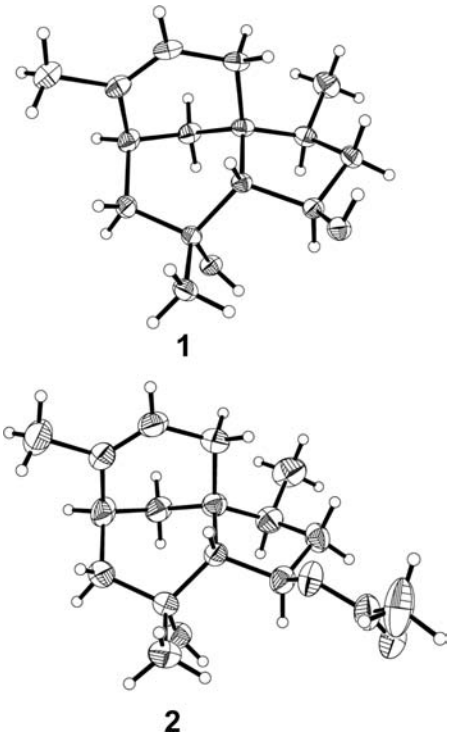


Figure 4. X-ray crystallographic structures of penicibilaenes A (**1**) and B (**2**).

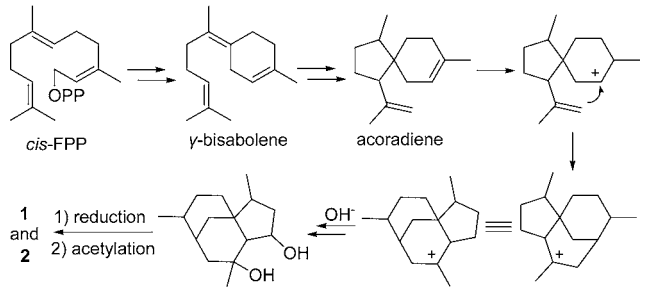
correlation from H-4 to C-1′ located the acetylation at 4-OH. HR-ESI-MS data for **2** revealed an expected adduct ion peak at *m*/*z* 301.1776 ([M + Na]$^+$, calcd 301.1774),[10] with 42 mass units more than that of **1**, providing the molecular formula $C_{17}H_{26}O_3$ for **2**. COSY and HMBC experiments (Figure 2), as well as NOESY data (Supporting Information), confirmed that **2** had the same connectivity and stereochemistry as **1**. An X-ray crystallographic experiment[11] further confirmed the structure and relative configuration of **2** as depicted (Figure 4). The Cu Kα radiation for the X-ray diffraction with the refined Flack parameter of 0.0(8) allowed the assignment of the absolute configuration of all the stereogenic centers in **2** as 1*R*, 2*S*, 4*R*, 5*R*, 6*R*, and 8*R*, same as that of **1**. Based on the above evidence, the structure of compound **2** was elucidated and it was named penicibilaene B.

Compounds **1** and **2** could be clearly detected from the culture extract of *P. bilaiae* MA-267 by TLC analysis (Figure S15 in the Supporting Information), thus providing solid evidence to rule out the possibility of artifactual nature of both compounds.

A plausible biosynthetic pathway for compounds **1** and **2** is proposed as shown in Scheme 1. In this pathway, γ-bisabolene is presumed to be a key intermediate synthesized from *cis*-farnesyl pyrophosphate (*cis*-FPP). Stereospecific and electronically favored cyclization involving the double bonds in γ-bisabolene could furnish acoradiene, another key intermediate. Compounds **1** and **2** are likely produced from acoradiene by a Wagner−Meerwein rearrangement,[13] followed by a series of modification reactions such as hydroxylation, reduction, and acetylation. This pathway is consistent with that presented by Tamiya and Sorensen in the synthesis of hispidospermidin, which was guided by the postulated biogenesis.[14]

Compounds **1** and **2** were tested against human- and aquapathogenic microbes *Aeromonas hydrophila*, *Edwardsiella tarda*,

Scheme 1. Proposed Biosynthetic Pathway for 1 and 2



Escherichia coli, *Staphylococcus aureus*, *Vibrio alginolyticus*, *V. anguillarum*, *V. harveyi*, and *V. parahemolyticus*.[15] None of them displayed potent activity against these strains (MIC > 64 μg/mL). Compounds **1** and **2** were also tested against plant pathogenic fungi *Alternaria brassicae*, *Colletotrichum gloeosporioides*, *Fusarium graminearum*, and *Gaeumannomyces graminis*. Both of them exhibited selective activity against *C. gloeosporioides* with MIC values of 1.0 and 0.125 μg/mL, respectively, the latter of which is better than that of the positive control, zeocin (MIC 0.25 μg/mL). This result indicated that compounds **1** and **2** are responsible for the activity against *C. gloeosporioides* during the preliminary assay of the crude extract, and acetylation of 4-OH likely enhanced the activity.

In conclusion, we isolated and identified two new sesquiterpenes penicibilaenes A and B (**1** and **2**) from the rarely studied fungus *P. bilaiae*, which was obtained from the rhizospheric soil of the marine mangrove plant *Lumnitzera racemosa*. These compounds contain a 2,6,9-trimethyltricyclo-[6.3.1.0^{1,5}]dodecane skeleton (named penicibilaene), which, to our knowledge, has only appeared as a subunit in a more complex structure (hispidospermidin).[12] The discovery of penicibilaene sesquiterpenes (**1** and **2**) might provide new targets for synthetic chemists, while the proposed biosynthetic pathway might be helpful in the total synthesis of these intriguing structures. Compounds **1** and **2** may prove useful as antifungal agents against the plant pathogen *C. gloeosporioides*, or this template may be used as a starting point for a medicinal chemistry program aimed at enhancing the antifungal potency.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, fungal material, extraction and isolation, X-ray crystallographic analysis, antimicrobial assay, and copies of 1D and 2D NMR spectra of compounds **1** and **2**. 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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(10) (a) Penicibilaene A (**1**): initially obtained as colorless amorphous powder, and crystals were obtained by slow evaporation in a mixture of MeOH−$CHCl_3$ (3:2) after 5 weeks; mp 171−173 °C; $[\alpha]_D^{25}$ +73.6 (*c* 0.19, MeOH); UV (MeOH) λ_{max} (log ε) 203 (3.67) nm; ^{1}H and ^{13}C NMR data, see Table 1; ESI-MS *m/z* 259 $[M + Na]^+$; HRESIMS *m/z* 259.1668 $[M + Na]^+$ (calcd for $C_{15}H_{24}O_2Na$, 259.1669). (b) Penicibilaene B (**2**): colorless crystals (MeOH); mp 138−140 °C; $[\alpha]_D^{25}$ +19.3 (*c* 1.1, MeOH); UV (MeOH) λ_{max} (log ε) 202 (3.56) nm; ^{1}H and ^{13}C NMR data, see Table 1; ESI-MS *m/z* 301 $[M + Na]^+$; HRESIMS *m/z* 301.1776 $[M + Na]^+$ (calcd for $C_{17}H_{26}O_3Na$, 301.1774).
(11) (a) Crystal data for penicibilaene A (**1**): $C_{15}H_{24}O_2$, FW = 236.34, monoclinic space group, *P*2(1), unit cell dimensions *a* = 9.1715(7)Å, *b* = 6.5653(4) Å, *c* = 11.0048(9) Å, *V* = 661.75(8) Å^3, α = γ = 90°, β = 92.9750(10), *Z* = 2, d_{calcd} = 1.186 mg/m^3, crystal dimensions 0.40 × 0.32 × 0.30 mm, μ = 0.596 mm^{-1}, *F*(000) = 260. The 2230 measurements yielded 1490 independent reflections after equivalent data were averaged, and Lorentz and polarization corrections were applied. The final refinement gave R_1 = 0.0339 and wR_2 = 0.0864 [$I > 2\sigma(I)$]. The absolute structure parameter was 0.0(3). (b) Crystal data for penicibilaene B (**2**): $C_{17}H_{26}O_3$, FW = 278.38, monoclinic space group, *P*2(1), unit cell dimensions *a* = 6.9819(7) Å, *b* = 7.4115(6) Å, *c* = 15.4050(11) Å, *V* = 795.85(12) Å3, α = γ = 90°, β = 93.279(6), *Z* = 2, d_{calcd} = 1.162 mg/m^3, crystal dimensions 0.18 × 0.16 × 0.12 mm, μ = 0.618 mm^{-1}, *F*(000) = 304. The 2530 measurements yielded 1997 independent reflections after equivalent data were averaged, and Lorentz and polarization corrections were applied. The final refinement gave R_1 = 0.0683 and wR_2 = 0.1234 [$I > 2\sigma(I)$]. The absolute structure parameter was 0.0(8).Crystallographic data for compounds **1** and **2** have been deposited at the Cambridge Crystallographic Data Centre, with deposition Nos. CCDC 1018665 and CCDC 1018666, respectively. 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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