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(±)-Sinensilactam A, a Pair of Rare Hybrid Metabolites with Smad3 Phosphorylation Inhibition from *Ganoderma sinensis*

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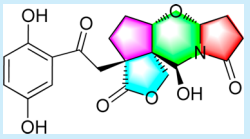
Supporting Information

ABSTRACT: (+)- and (−)-Sinensilactam A (**1**), novel hybrid metabolites possessing a unique 2*H*-pyrrolo[2,1-*b*][1,3]oxazin-6(7*H*)-one ring system, were isolated from the fruit bodies of *Ganoderma sinensis*. The structures of these substances and absolute configurations at their stereocenters were assigned using spectroscopic and computational methods along with X-ray crystallographic analysis. A plausible pathway for the biosynthesis of **1** is proposed. (−)-**1** was found to be a Smad3 phosphorylation inhibitor in TGF-β1 induced human renal proximal tubular cells.



Ganoderma is a genus of polypore fungi that grows on wood, the majority of which are found in tropical regions.[1] Several species of the genus *Ganoderma*, known as lingzhi in Chinese, are renowned for their medical value.[2,3] Lingzhi mushrooms are complex in that they are observed in more than one form or even as distinctly separate species. The extensive medicinal use of lingzhi mushrooms for the prevention and treatment of various diseases in East Asian countries has attracted significant attention in recent decades.[4,5] An updated search by using SciFinder uncovered 14,638 literature citations to *Ganoderma*. Compounds, including triterpenoids and polysaccharides, have been isolated as bioactive constituents of several *Ganoderma* species.[6] Because it is well-known that members of these natural product families are also present in many other organisms, a question exists about the presence of other unique families in the genus *Ganoderma* that are responsible for its multiple beneficial health effects.

Ganomycins A and B, a new compound class, were isolated from *G. pfeifferi* in 2000.[7] Following this report, few other observations related to the isolation of members of this compound class from *Ganoderma* species have been described. In 2013, we isolated and characterized a novel rotary-door-like meroterpenoid, named lingzhiol, from *G. lucidum* and showed that it is a potent Smad3 phosphorylation inhibitor.[8] This finding attracted significant attention.[9,10]

In continuing efforts focused on the search for biologically active natural products from lingzhi mushrooms,[8,11,12] we investigated *G. sinensis*. This mushroom is a fungal species described in *Pharmacopoeia of People's Republic of China* (2000 edition) and distributed in the eastern and southern regions of China. Many earlier studies of this traditional medicine revealed that it contains polysaccharides, triterpenoids, alkaloids, fatty acids, nucleotides, proteins, peptides, trace elements, sterols, and ganosinensins A−C, the latter of which are hybrids of a triterpenoid and a prenylated phenols.[6,13,14] Despite these observations, the presence of meroterpenoids in this species remains largely unknown. Our recent efforts focusing on lingzhi meroterpenoids from *G. sinensis* led to the isolation of a pair of enantiomers of a meroterpenoidal hybrid metabolite, called sinensilactam A, which contains a rare 2*H*-pyrrolo[2,1-*b*][1,3]-oxazin-6(7*H*)-ring system. Below we describe the structure elucidation and biological evaluation of sinensilactam A.

(±)-Sinensilactam A (**1**),[15] obtained from *G. sinensis* as colorless crystals (MeOH), has the molecular formula $C_{20}H_{21}NO_8$ (11 degrees of unsaturation), based on analysis of its HRESIMS, ^{13}C NMR, and DEPT spectra. The ^1H NMR spectrum (Table 1) of **1** contains signals for a typical ABX spin system [δ_H 7.22 (1H, d, J = 3.0 Hz, H-3), δ_H 6.99 (1H, dd, J = 8.9, 3.0 Hz, H-5), δ_H 6.83 (1H, d, J = 8.9 Hz, H-6)], suggesting the presence of a 1,2,4-trisubstituted benzene ring. The ^{13}C NMR and DEPT spectra indicate that **1** is comprised of 20 carbons ascribed to 6 methylenes (one oxygenated), 6 methines (three sp^2, three oxygenated), and 8 quaternary carbons (one ketone, two carbonyls, three olefinic including two oxygenated, and two aliphatic). The $^1H-^1H$ COSY spectrum (Figure 2) of this substance shows that H-5/H-6, H-4′/H-5′/H-6′, H-8′/8′-OH, and H-3″/H-4″/H-5″ fragments exist.

The planar structure of **1** (Figure 1) was constructed mainly based on the results of 2D NMR experiments. The HMBC correlations of H-3, H-6/C-1′, and H-2′/C-2, C-1′ suggest that the arene ring (part A) in this substance is linked to C-1′ by C-

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Table 1. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) Data of **1** in DMSO-d_6 (δ in ppm, J in Hz)

position	δ_H	δ_C
1		153.3 s
2		120.0 s
3	7.22 (d, 3.0)	114.6 d
4		149.5 s
5	6.99 (dd, 8.9, 3.0)	124.3 d
6	6.83 (d, 8.9)	118.5 d
1′		201.6 s
2′	a 3.85 (d, 19.1)	45.4 t
	b 3.41 (d, 19.1)	
3′		51.3 s
4′	a 1.85 (dd, 13.7, 6.1)	33.9 t
	b 1.80 (dd, 13.7, 6.2)	
5′	a 1.90 (m)	25.0 t
	b 1.55 (m)	
6′	4.32 (dd, 12.1, 5.7)	77.1 d
7′		48.2 s
8′	5.45 (brs)	75.9 d
9′	a 4.24 (d, 10.2)	69.2 t
	b 3.76 (d, 10.2)	
10′		181.4 s
2″		172.6 s
3″	a 2.39 (d, 17.7)	29.3 t
	b 2.29 (d, 17.7)	
4″	a 2.25 (overlap)	23.5 t
	b 1.73 (m)	
5″	5.25 (dd, 6.3, 3.7)	84.5 d
1-OH	10.7 (s)	
4-OH	9.20 (s)	
8′-OH	6.53 (brs)	

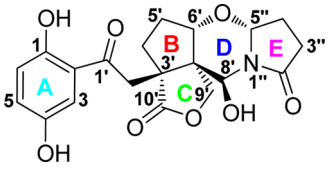

Figure 1. Structure of (−)-**1** (3′*S*,6′*S*,7′*R*,8′*S*,5″*R*).

2. The presence of a cyclopentane ring in part B is consistent with the observed $^{1}H-^{1}H$ COSY correlations of H-4′/H-5′/H-6′ and the HMBC correlations of H-4′/C-3′, C-7′ and H-5′/C-3′, C-7′. Moreover, HMBC correlations of H-6′/C-9′, H-4′/C-10′, and H-9′/C-3′, C-6′, C-7′, C-10′ suggest that part B in **1** is comprised of a cyclopenta[*c*]furolactone motif. Correlations are observed in the HMBC spectrum from H-3″, H-4″, and H-5″ to C-2″ (δ_C 172.6), and $^{1}H-^{1}H$ COSY correlations are observed for H-3″/H-4″/H-5″. These findings indicate the presence of the 2-pyrrolidinone unit in **1** (Figure 2, green line), a proposal that is supported by analysis of the molecular composition and chemical shift of C-5″ (δ_C 84.5).

Apart from the 10 degrees of unsaturation associated with one arene ring, a cyclopenta[*c*]furolactone structure, 2-pyrrolidinone moiety, and one ketone carbonyl, the remaining degree of unsaturation is attributed to an additional ring in **1**. HMBC correlations of H-6′/C-5″ and H-8′ (δ_H 5.45)/C-3′, C-6′ (δ_C 77.1), C-9′, C-2″ suggest that C-6′ is connected to C-5″ via an oxygen bridge as part of a six-membered ring.

The relative configurations at the stereogenic centers in **1** were assigned by analysis of ROESY data (Figure 3).

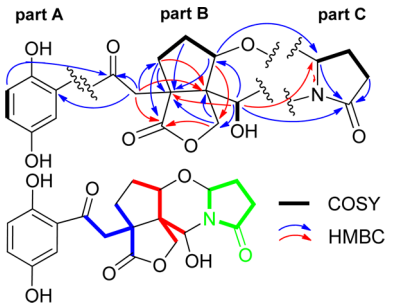

Figure 2. Key COSY and HMBC correlations of **1**; blue and red in **1** represent two independent isoprenyl moieties.

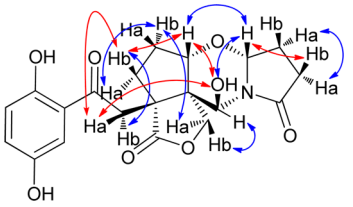

Figure 3. Key ROESY correlations of **1**.

Specifically, ROESY correlations between 8′-OH (δ_H 6.53)/H-6′ (δ_H 4.32), H-5″ (δ_H 5.25), and H-6′/H-5″ show that 8′-OH/H-6′/H-5″ are oriented on the same side of ring D. In addition, correlations between Ha-9′ (δ_H 4.24)/Hb-5′ (δ_H 1.55) and Hb-9′ (δ_H 3.76)/H-8′ (δ_H 5.45) enable assignment of the relative configuration of the spiro-juncture at C-7′. Correlations between 8′-OH/Ha-2′ (δ_H 3.85), Ha-4′ (δ_H 1.85)/Hb-5′, and Hb-4′ (δ_H 1.80)/H-2′ show that the assigned configuration of C-3′ is correct. In this circumstance, the observed ROESY correlation of H-6′/Hb-4′ (δ_H 1.73) enables establishment of the relative configuration at C-6′. The structural conclusions drawn from inspection of the NMR data are unambiguously confirmed by using X-ray diffraction analysis of (±)-**1** (Figure 4).

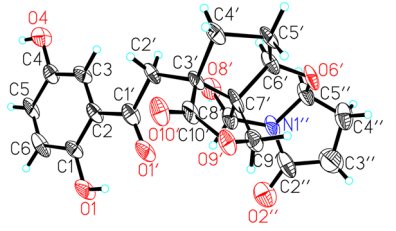

Figure 4. Ortep plot of the X-ray crystallographic data for (±)-**1**.

(±)-Sinensilactam A (**1**) was isolated in an enantiomerically impure form. Separation by using chiral-phase HPLC yielded (+)-**1** and (−)-**1** in a ratio of 1:1.2. Owing to difficulties associated with obtaining single crystals of either (+)-**1** or (−)-**1**, assignments of absolute configurations at the stereogenic centers in these substances were made by comparing the M06/TZVP calculated ECD spectrum of the (3′*R*,6′*R*,7′*S*,8′*R*,5″*S*)-isomer with the experimental spectra of both enantiomers. The results suggest that the absolute configuration at the stereocenters in (+)-**1** are 3′*R*,6′*R*,7′*S*,8′*R*,5″*S* (Figure 5).

Analysis of **1** (Figure 2) demonstrates that, apart from the dihydroquinone ring, its structure is comprised of an aryl linked monoterpenoid and a pyrrolidinone fragment. This conclusion

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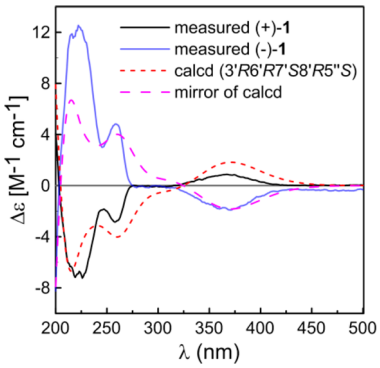
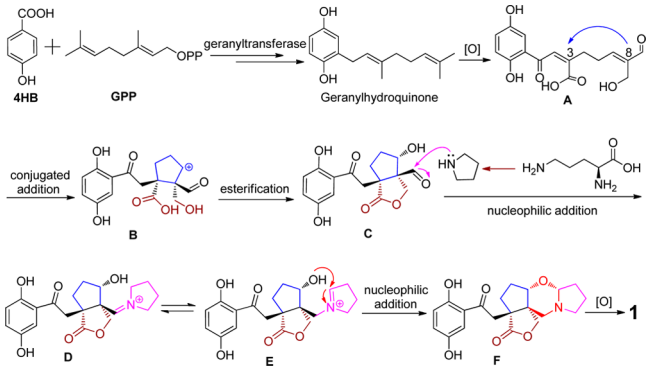


Figure 5. Comparison of M06/TZVP calculated ECD spectrum for (3′*R*,6′*R*,7′*S*,8′*R*,5″*S*)-isomer with the experimental spectra of (+)-**1** and (−)-**1** in MeOH. σ = 0.37 eV; shift = +8 nm; scaled by 1.0.

makes it possible to propose that sinensilactam A is biogenetically derived by a route (Scheme 1) that is a hybrid

Scheme 1. Plausible Pathway for the Biogenesis of 1



of the shikimic acid, mevalonic acid, and amino acid pathways (Scheme 1). Fungi and plants biosynthesize aromatic compounds such as 4-hydroxybenzonic acid (4HB) via the shikimic acid pathway[16] starting with erythrose-4-phosphate and phosphoenolpyruvate.[17] The monoterpene precursor, geranyl diphosphate (GPP), derived from isopentenyl diphosphate produced in fungi by the mevalonic acid pathway, then joins with 4HB in a reaction promoted by geranyltransferase.[18] The resulting geranylhydroquninone is then oxidized to generate A, which cyclizes via a conjugated addition to generate B. Esterification of B then yields C. The final and interesting phase of the biosynthesis of **1** involves formation of the pyrrolidinone moiety through the amino acid synthesis pathway. Pyrrolidine is usually derived from L-ornithine.[19] Ring D of **1** is formed by nucleophilic addition followed by oxidation of monomer F to give sinensilactam A. Of note, **1** was isolated as a racemate, this phenomenon was also observed for meroterpenoids present in other *Ganoderma* species,[8,9,12] which allows us to tentatively hypothesize that these racemates may simply be the early stage intermediates of random oxidation by an enzyme prior to formation of high-molecular weight end products. However, this needs further investigation.

Inspired by ethnopharmacological knowledge, our recent attention has focused on a search for substances that are active against renal fibrosis. Among various signaling pathways of renal fibrosis is TGF-β/Smads, which is considered to be the key profibrotic pathway. The results of *in vitro* and *in vivo* experiments have shown that phosphorylation of Smad3 (p-Smad3) induced by TGF-β is closely associated with fibrotic cascades.[20] Therefore, compounds that inhibit p-Smad3 are of great interest. Unfortunately, except for the drugs SIS3 and halofuginone,[21,22] selective inhibitors of Smad3 phosphorylation are scarce. Recently, the discovery of GQ5 in our laboratory represents another milestone in the fibrotic field.[23]

Considering that *G. sinensis* is utilized in traditional Chinese medicine for the treatment of a wide variety of disorders, including chronic kidney disease, we have tested the inhibitory effects of the enantiomers from *G. sinensis* on p-Smads. As shown by the results of Western blot (Figure 6 and Supporting

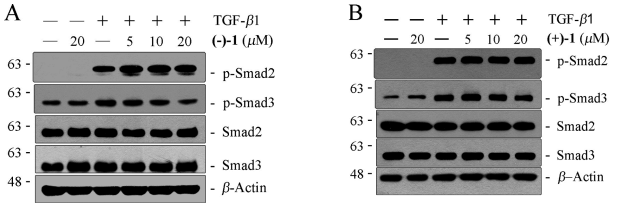


Figure 6. Compounds (−)-**1** (A) and (+)-**1** (B) selectively block TGF-β1-mediated Smad3 but not Smad2 phosphorylation in a dose-dependent manner. HKC-8 cells were treated with TGF-β1 (5 ng/mL) for 3 h in the absence or presence of different doses of (+)-**1** and (−)-**1** as indicated. Cell lysates after various treatments as indicated were immunoblotted with antibodies against phosphorylated Smad2, phosphorylated Smad3, Smad2, Smad3, and β-actin.

Information), phosphorylation of Smad3 but not of Smad2 is inhibited by (+)-**1** and (−)-**1**, indicating their selectivity toward p-Smad3. Besides, the results show that (−)-**1** appears to be more potent than (+)-**1**. Finally, to secure this result, the MTT exclusion test was carried out, which shows that either (+)-**1** or (−)-**1** is not toxic in human renal proximal tubular cells at concentrations of 2.5 to 20 μM (Supporting Information).

■ ASSOCIATED CONTENT

Supporting Information

1D, 2D NMR, and MS spectra, detailed isolation procedures, crystallographic data, computational and bioassay methods. This material is available free of charge via Internet at http://pubs.acs.org.

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Author Contributions

⊥Q.L., L.T., and L.D. contributed equally.

Notes

The authors declare no competing financial interest.

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(15) Sinensilactam A (**1**): colorless crystal; $[\alpha]_D^{20.3}$ −16.6 (*c* 0.53, MeOH); UV (MeOH) λmax (log ε) 366 (3.48), 258 (3.76), 226 (4.05) nm; ESIMS *m*/*z* 402 $[M-H]^-$, HRESIMS *m*/*z* 426.1159 $[M+Na]^+$ (calcd for $C_{20}H_{21}NO_8Na$, 426.1165). For ^1H and ^{13}C NMR data, see Table 1. $[\alpha]_D^{20.3}$ +35.7 (*c* 0.11, MeOH); CD (MeOH) $\Delta\varepsilon_{219}$ −7.13, $\Delta\varepsilon_{258}$ −2.84, $\Delta\varepsilon_{366}$ +0.88; (+)-**1**; $[\alpha]_D^{18.6}$ −33.2 (*c* 0.10, MeOH); CD (MeOH) $\Delta\varepsilon_{222}$ +12.5, $\Delta\varepsilon_{259}$ +4.81, $\Delta\varepsilon_{371}$ −1.91; (−)-**1**.
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