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Unusual constituents from the medicinal mushroom *Ganoderma lingzhi*[†]

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Extensive studies have revealed that triterpenoids, meroterpenoids, and polysaccharides are the main constituents of the well-known traditional Chinese medicinal mushroom *Ganoderma*. In this study, we report seven previously undescribed sesquiterpenoids, including six gymnomitrans (**1–6**) and a novel type of sesquiterpenoid (**8**), together with a polyketide (**7**) and a known steroid (**9**) from the fruiting bodies of *Ganoderma lingzhi*, a fungus used as traditional medicine and food supplement in East Asia for ages. The structures of **1–8** were deduced by analysis of spectroscopic data, X-ray single crystal diffractions and TDDFT/ECD calculations. Compound **8** possessed an unusual 14(7–6)-cuparane scaffold. Compound **9** exhibited weak cytotoxicity against the five human cancer cell lines HL-60, MCF-7, SW480, A549, and SMMC-7721 with IC₅₀ values of 18.0–32.3 μ M. A simple structure-activity-relationship (SAR) investigation by acetylating the 5-OH of **9** (**9a**) suggested that the 5-OH is essential for its cytotoxicity. Additionally, the biosynthetic pathways for compounds **2** and **8** are discussed.

Introduction

Ganoderma have been used as traditional and folk medicines for thousands of years in East Asia. *Ganoderma* natural products have long been a hot topic for their health-preserving and therapeutic effects.^{1,2} The *Ganoderma lucidum* complex includes more than 23 described species.³ This genus has proved to be a prolific source of triterpenes, and to date, more than 400 examples have been reported.⁴ *Ganoderma* constituents have shown anti-tumour, anti-inflammatory, anti-microbial, acetylcholinesterase inhibitory,⁵ and pancreatic lipase inhibitory,⁶ and have emerging potency as anti-aging agents.⁷ Extensive studies have revealed that the polysaccharides and triterpenes are responsible for the anti-tumour and immunomodulatory functions.⁵ Notably, *Ganoderma* meroterpenoids have widely attracted attention in the past six years due to their intriguing structures and diverse biological activities.⁸ All these efforts have illuminated and enriched the chemical profile of *Ganoderma* secondary metabolites, which has made it possible for

Ganoderma to be developed as a regular drug rather than as a dietary supplement.^{9,10}

Sesquiterpenoids are the most abundant type of secondary metabolites in mushrooms.¹¹ However, in contrast to the attention that has been paid on the triterpenes, research on sesquiterpenes from *Ganoderma* fruiting bodies is still unfolding. The *Ganoderma* genome-sequencing, as part of the herb genomics plan, revealed that *Ganoderma* encodes terpene synthases for monoterpene, sesquiterpene, and diterpene backbones.¹² However, hitherto only fourteen sesquiterpenoids have been reported from the fermentation products of three different *Ganoderma* species,⁴ while only two other sesquiterpenes were isolated from *Ganoderma* fruiting bodies, *i.e.* ganosinensine¹³ and gymnomitrane-3 α ,5 α ,9 β ,15-tetrol.¹⁴ Besides, the first sesquiterpene ganosinensine was then regarded as a metabolite of the symbiotic bacteria of *G. sinense*.

Ganoderma lingzhi, widely distributed in China, Japan, and Korea, is a new species firstly proposed by Y.-C. Dai in 2012¹⁵ and then regarded as a later synonym of *G. sichuanense*, although there is still a controversy about the taxonomy between *G. lingzhi* and *G. sichuanense*.^{16,17} Comparing with the other *Ganoderma* species, the secondary metabolites of *G. lingzhi* have rarely been investigated. Y. M. Yan *et al.* reported six meroterpenoids with neural stem cells proliferation-promoting activity from this fungus.¹⁸

In order to explore diverse constituents for the identification of bioactive natural products from *Ganoderma*, a detailed chemical investigation on *G. lingzhi* led to the isolation of six rarely-encountered gymnomitrane-type sesquiterpenoids (**1–6**), an unusual type sesquiterpene (**8**), a polyketide (**7**) and an

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unusual ergosterol (**9**) from the low-polarity fractions of the crude extract (Fig. 1). Herein we report the isolation, structural characterization, biological activity, and feasible biosynthetic pathways of the compounds **1–9**.

Results and discussion

Compound **1**, colourless needles (MeOH), had a molecular formula of $C_{15}H_{22}O_2$ deduced by HREIMS analysis. The 1D NMR data (Tables 1 and 2) displayed signals for three methyl singlets, four sp^3 methylenes, a trisubstituted double bond, two sp^3 methines (one oxygenated carbon δ_C 77.6), an aldehyde group (δ_C 193.0), and three sp^3 quaternary carbons. The data of **1** showed similarity to those of (+)-5-hydroxybarbatenal,¹⁹ a gymnomitrane-type sesquiterpene isolated from the root of *Beilschmiedia tsangii*. The data also suggested a hydroxy substituted at C-10 in **1** rather than C-5 in (+)-5-hydroxybarbatenal. This change was supported by the mutual 1H - 1H COSY correlations between H-9 (δ_H 1.76, 1.61) and H-10 (δ_H 3.67), along with the HMBC correlations from Me-12 (δ_H 1.06) to C-10 (δ_C 77.6) (Fig. 2). The relative configuration of **1** was determined to be $2S^*,6R^*,7R^*,10R^*,11S^*$ in accordance with the diagnostic ROESY correlations of H-1 β /H₃-13/H₃-14, and H₃-12/H-2/H-10 (Fig. 3). The absolute configuration of **1** was unequivocally established as $2S,6R,7R,10R,11S$ by single crystal X-ray diffraction analysis (Fig. 4) with the Flack parameter = 0.09(6). Hence, the structure of **1** was established as shown in Fig. 1, and named as 10 α -hydroxy-gymnomitr-3-en-15-al.

Compound **2** was obtained as colourless crystals (MeOH). Its molecular formula was determined to be $C_{15}H_{22}O_2$ by HREIMS analysis with the ion peak at m/z 234.1617 (calcd for $C_{15}H_{22}O_2$ 234.1620), indicating the presence of five degrees of unsaturation. The planar structure of **2** was elucidated by interpretation of its NMR spectra. The 1D NMR spectra of **2** presented three

methyls (δ_H/δ_C : 0.94/20.0, 0.97/24.7, 1.11/27.4), four methylenes (δ_C 31.5, 32.7, 38.0, 74.2), two sp^3 methines (δ_C 53.9, 88.8), two olefinic carbons (δ_C 130.2, 142.3), and four sp^3 quaternary carbons (δ_C 43.6, 52.4, 64.5, 69.7) (Tables 1 and 2). The aforementioned data indicated that **2** is a sesquiterpene derivative harbouring a gymnomitrane skeleton analogous to that of **1**. In addition to the tricyclic system and one double bond, the remaining one degree of unsaturation revealed the existence of an additional ring. Analysis of the 2D NMR spectra of **2** helped to establish its structure unambiguously. The HMBC correlations from H₃-13 (δ_H 0.97) to C-8 (δ_C 142.3) revealed that the double bond was located between C-8 and C-9. Additionally, the HMBC correlations from H-4 (δ_H 1.86, 1.48) to C-3 (δ_C 69.7) and C-15 (δ_C 74.2), and from H-15 (δ_H 3.43, 3.27) to C-10 (δ_C 88.8) suggested an ether linkage between C-10 and C-15, and a hydroxy group substituted at C-3 (Fig. 2). The key ROESY correlations between H₃-12 (δ_H 1.11)/H-10 (δ_H 4.11), together with biosynthetic comparison with the structure of **1** and the structure inflexibility of **2**, the absolute configuration of **2** was assigned to be $2S,3R,6R,7R,10R,11S$. The above assignment indicated the structure of **2** as a unique boat-like molecule bearing a rigid structure unprecedented in the gymnomitrane family, it was given the name 10 α ,15-epoxy-gymnomitr-8-en-3 β -ol.

Compound **3** was isolated as a white amorphous powder. The molecular formula was deduced from the sodium adduct ion peak at m/z 257.1514 (calcd for $C_{15}H_{22}O_2Na$, 257.1512) as $C_{15}H_{22}O_2$ in the HRESIMS analysis. The ^{13}C NMR spectrum (Table 2) showed signals for 15 carbons, including three methyls, five methylenes (one was oxygenated δ_C 65.6), one sp^3 methines, three sp^3 quaternary carbons, and an α,β -unsaturated ketone group (δ_C 169.5, 122.3, 205.5). The data shared striking similarity with those of the known compound ganosinensine isolated from *G. sinense*,¹³ indicating that **3** was also a gymnomitrane-type sesquiterpene. The structural difference between ganosinensine and **3** was that the C-9 in **3** remained unoxygenated, supported by the 1H - 1H COSY correlations of H-8 (δ_H 1.46, 1.17)/H-9 (δ_H 1.53, 1.43)/H-10 (δ_H 1.59, 1.36) (Fig. 2) and HRESIMS data. The configuration of **3** was identical with that of **1** by analysis of the ROESY spectrum (Fig. 3). Thus, compound **3** was determined as shown in Fig. 1, and named as 15-hydroxy-gymnomitr-3-en-5-one.

The HRESIMS analysis of **4** suggested the molecular formula was $C_{15}H_{20}O_4$ (m/z 263.1291 [$M - H$]⁻, calcd for $C_{15}H_{19}O_4$, 263.1289), appropriate for six degrees of unsaturation. The 1H and ^{13}C NMR data (Tables 1 and 2) showed representative chemical shifts for a gymnomitrane skeleton: two methyl singlets (δ_H 1.13, 1.21; δ_C 24.7, 30.6), three sp^3 quaternary carbons (δ_C 47.0, 61.6, 71.6), two carbonyl groups (δ_C 170.7, 178.7), one tri-substituted double bond (δ_C 140.3, 137.7; δ_H 6.80). The HMBC correlations from H-2 (δ_H 2.61) to C-3 (δ_C 137.7) and C-4 (δ_C 140.3) allowed the assignment of C-3/C-4 as a double bond (Fig. 2). The gymnomitrane scaffold and the double bond accounted for four out of six degrees of unsaturation. The remaining two degrees of unsaturation were assigned to two carboxylic groups at C-13 and C-15, which was supported by the HMBC correlations from H-8 (δ_H 2.22) to C-13

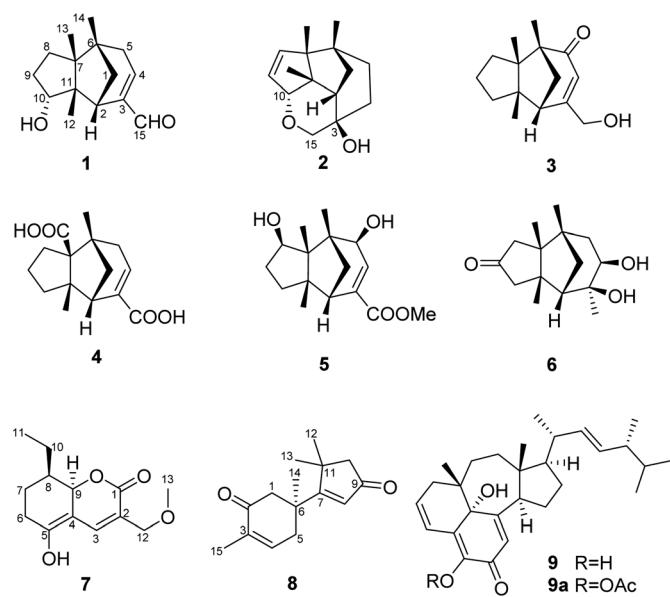


Fig. 1 Structures of compounds **1–9**, and **9a**.



Table 1 ^1H NMR data of compounds 1–8 (600 MHz)

| No. | 1 ^b | 2 ^a | 3 ^b | 4 ^a | 5 ^a | 6 ^b |
|------|---|--|---|--|---|--|
| 1 | 2.05, dd (11.8, 3.5), 1.37, d (11.8) | 1.77, dd (10.0, 2.5), 1.80, dd (10.0, 2.5) | 2.01, dd (11.5, 4.5), 1.87, d (11.5) | 2.79, dd (11.0, 5.0), 1.42, d (11.0) | 1.67, dd (11.8, 4.5), 1.60, d (11.8) | 1.59, ddd (12.0, 5.0), 2.8), 2.11, d (12.0) |
| 2 | 2.59, d (3.5) | 1.61, t (2.5) | 2.11, d (4.5) | 2.61, d (5.0) | 2.51, d (4.5) | 1.85, d (5.0) |
| 4 | 6.73, t-like (2.5) | 1.86, overlapped, 1.48, ddd (16.8, 7.6, 5.2) | 6.04, br. s | 6.80, dd (4.0, 3.0) | 6.88, d (4.0) | 3.31, dd (11.2, 6.3) |
| 5 | 2.55, dd (22.0, 2.5), 2.24, dd (22.0, 2.5) | 1.84, overlapped, 1.32, ddd (16.8, 13.3, 8.0) | | 2.49, dd (21.0, 4.0), 2.16, dd (21.0, 3.0) | 3.95, d (4.0) | 1.82, dd (14.0, 6.3), 1.46, dd (14.0, 11.2) |
| 8 | 1.50, m, 1.23, m | 5.65, d (5.8) | 1.46, m, 1.17, overlapped | 2.22, ddd (14.0, 9.5, 8.0), 1.64, ddd (14.0, 6.0, 4.0) | 3.74, overlapped | 2.61, d (19.7), 1.89, d (19.7) |
| 9 | 1.76, m, 1.61, m | 5.60, dd (5.8, 2.1) | 1.53, m, 1.43, m | 1.60, m, 1.54, m | 1.74, m | |
| 10 | 3.67, t-like (5.0) | 4.11, d (2.1) | 1.59, overlapped, 1.36, m | 1.69, ddd (14.0, 7.0, 4.0), 1.25, m | 1.27, m, 1.14, overlapped | 3.00, d (19.8), 2.13, d (19.8) |
| 12 | 1.06, s | 1.11, s | 1.17, s | 1.21, s | 1.12, s | 1.22, s |
| 13 | 0.94, s | 0.97, s | 0.98, s | | 1.05, s | 1.01, s |
| 14 | 0.99, s | 0.94, s | 1.07, s | 1.13, s | 0.93, s | 0.90, s |
| 15 | 9.45, s | 3.43, d (10.0), 3.27, d (10.0) | 4.35, br. dd (17.0, 6.0), 4.25, br. dd (17.0, 6.0) | | | 1.38, s |
| MeO- | | | | | 3.74, s | |

^a Measured in CD_3OD . ^b Measured in CDCl_3 .

(δ_{C} 178.7), and from H-2 (δ_{H} 2.61) and H-4 (δ_{H} 6.80) to C-15 (δ_{C} 170.7) (Fig. 2). The relative stereochemistry of **4** is identical to that of **1** by analysis of the ROESY spectrum (Fig. 3). Hence, **4** was named gymnomitr-3-ene-13,15-dioic acid.

The 1D NMR data of **5** (Tables 1 and 2) exhibited four methyl singlets (one methoxy group), three sp^3 methylenes, three sp^3 methines (two oxygenated), one tri-substituted double bond, three sp^3 quaternary carbons, and one carbonyl group. The data displayed similarity to those of **1–4**, suggesting the gymnomitrane skeleton of **5**. Compared to compound **1**, two hydroxy groups substituted at C-5 and C-8 in **5**, rather than a hydroxy group at C-10 in **1**. The changes were supported by the HMBC correlations from H₃-13 (δ_{H} 1.05) to C-8 (δ_{C} 76.2), H₃-14 (δ_{H} 0.93) to C-5 (δ_{C} 71.6), and H₃-12 (δ_{H} 1.12) to the methylene C-10 (δ_{C}

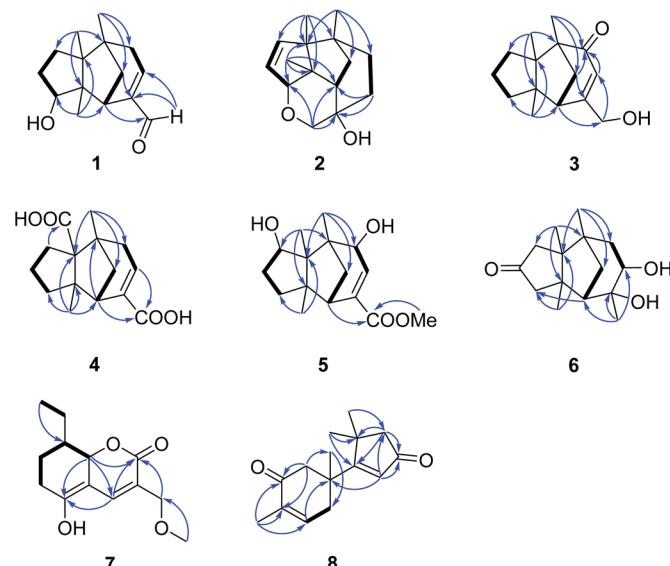
33.6) (Fig. 2). Furthermore, the HMBC correlation from the methoxy singlet at δ_{H} 3.74 to the carbonyl group δ_{C} 169.1 suggested the presence of a methyl ester group at C-15 in compound **5** (Fig. 2). The above assignments are consistent with the molecular formula $\text{C}_{16}\text{H}_{24}\text{O}_4$, which was determined by HRESIMS data. The relative configurations of C-5 and C-8 were assigned as *S** and *R**, respectively, by the key ROESY correlations between H-5 (δ_{H} 3.95) and H-8 (δ_{H} 3.74) (Fig. 3). The absolute configuration of **5** was unequivocally settled by single crystal X-ray diffraction analysis as 2*S*,5*S*,6*R*,7*S*,8*R*,11*S* (Fig. 5). Thus, compound **5** was identified as methyl 5 β ,8 β -dihydroxygymnomitr-3-en-15-oate.

Compounds **6** and **7** were isolated as an inseparable mixture. The NMR spectra of the mixture presented complicated but

Table 2 ^{13}C NMR Data of Compounds 1–8 (150 MHz)

| No. | 1 ^b | 2 ^a | 3 ^b | 4 ^a | 5 ^a | 6 ^b | 7 ^b | 8 ^b |
|------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| 1 | 42.0, CH_2 | 38.0, CH_2 | 47.1, CH_2 | 43.7, CH_2 | 38.3, CH_2 | 37.4, CH_2 | 178.5, C | 50.4, CH_2 |
| 2 | 42.1, CH | 53.9, CH | 49.0, CH | 47.1, CH | 46.5, CH | 57.9, CH | 125.1, C | 197.8, C |
| 3 | 147.6, C | 69.7, C | 169.5, C | 137.7, C | 140.0, C | 72.6, C | 152.7, CH | 135.4, C |
| 4 | 151.0, CH | 31.5, CH_2 | 122.3, CH | 140.3, CH | 139.0, CH | 69.5, CH | 124.2, C | 142.3, CH |
| 5 | 41.9, CH_2 | 32.7, CH_2 | 205.5, C | 42.2, CH_2 | 71.6, CH | 44.6, CH_2 | 166.3, C | 38.4, CH_2 |
| 6 | 44.5, C | 43.6, C | 60.4, C | 47.0, C | 47.6, C | 45.4, C | 28.0, CH_2 | 43.2, C |
| 7 | 55.6, C | 64.5, C | 55.3, C | 71.6, C | 57.0, C | 50.1, C | 22.2, CH_2 | 190.4, C |
| 8 | 32.7, CH_2 | 142.3, CH | 37.9, CH_2 | 31.6, CH_2 | 76.2, CH | 48.4, CH_2 | 40.2, CH | 130.0, CH |
| 9 | 35.2, CH_2 | 130.2, CH | 26.9, CH_2 | 28.3, CH_2 | 35.4, CH_2 | 219.6, C | 62.6, CH | 207.1, C |
| 10 | 77.6, CH | 88.8, CH | 39.2, CH_2 | 40.1, CH_2 | 33.6, CH_2 | 50.5, CH_2 | 23.7, CH_2 | 55.2, CH_2 |
| 11 | 61.3, C | 52.4, C | 56.9, C | 61.6, C | 59.3, C | 49.7, C | 11.9, CH_3 | 45.1, C |
| 12 | 19.6, CH_3 | 27.4, CH_3 | 28.1, CH_3 | 30.6, CH_3 | 27.7, CH_3 | 30.9, CH_3 | 66.2, CH_2 | 29.2, CH_3 |
| 13 | 24.4, CH_3 | 24.7, CH_3 | 22.8, CH_3 | 178.7, C | 20.0, CH_3 | 24.5, CH_3 | | 29.4, CH_3 |
| 14 | 24.2, CH_3 | 20.0, CH_3 | 17.6, CH_3 | 24.7, CH_3 | 15.9, CH_3 | 23.3, CH_3 | | 27.6, CH_3 |
| 15 | 193.0, CH | 74.2, CH_2 | 65.6, CH_2 | 170.7, C | 169.1, C | 27.7, CH_3 | | 15.4, CH_3 |
| MeO- | | | | | 52.5, CH_3 | | 59.1, CH_3 | |

^a Measured in CD_3OD . ^b Measured in CDCl_3 .

Fig. 2 Selected HMBC and ^1H - ^1H COSY correlations of 1-8.

clear signals which could be assigned to two sets with the aid of HSQC, HMBC, and ^1H - ^1H COSY spectra. The first group of signals designated to 6 included four methyl singlets (δ_{H} 0.90, 1.01, 1.22, 1.38; δ_{C} 23.3, 24.5, 30.9, 27.7), four methylenes, two methines (one attached to an oxygen atom), and five quaternary carbons (one carbonyl and one was oxygenated) (Tables 1 and 2). The above-mentioned data are in agreement with a gymnomitrane skeleton substituted by two hydroxy groups at C-3 (δ_{C} 72.6), C-4 (δ_{C} 69.5), and a carbonyl group at C-9 (δ_{C} 219.6C), which were supported by the HMBC correlations from H_3 -15 (δ_{H} 1.38) to C-3 and C-4, and from H-8 (δ_{H} 2.61, 1.89), H-10 (δ_{H} 3.00, 2.13) to C-9 (δ_{C} 219.6) (Fig. 2). The assignments were in accordance with the molecular formula of $\text{C}_{15}\text{H}_{24}\text{O}_3$ generated by

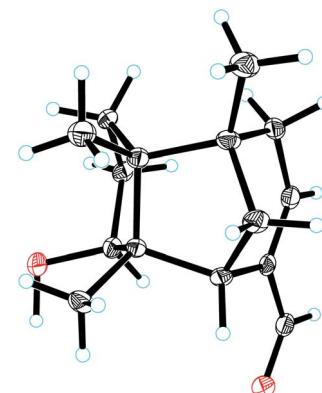


Fig. 4 ORTEP drawing of compound 1.

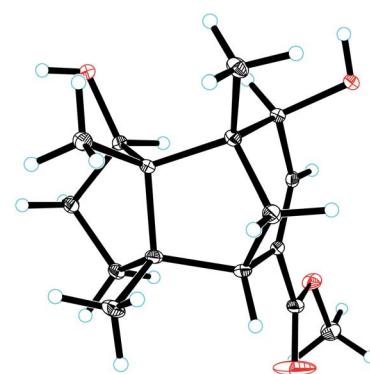


Fig. 5 ORTEP drawing of compound 5.

HREIMS ion peak at m/z 252.1724 $[\text{M}]^+$ (calcd for 252.1725). As for the relative configuration of 6, both of the hydroxy groups at C-3 and C-4 were assigned to be β oriented by the diagnostic

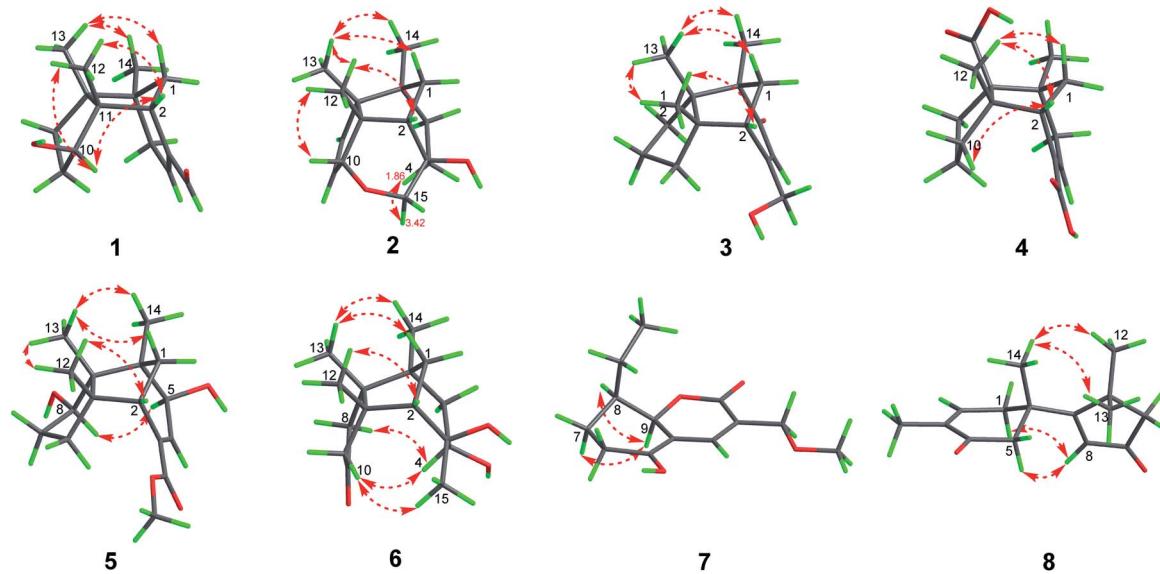


Fig. 3 Selected ROESY correlations of 1-8.



ROESY correlations between H-8 α (δ_H 2.61) and H-4 (δ_H 3.31), H-10 α (δ_H 2.98) and H-4, and between H₃-15 (δ_H 1.38) and H-10 α (Fig. 4). Thus, compound **6** was established to be 3 β ,4 β -dihydroxy-gymnomitr-9-one.

The other group of signals included a methyl triplet (δ_H 1.01; δ_C 11.9), a methoxy group (δ_H 3.44; δ_C 59.1), four methylenes (one oxygenated), three methines (one oxygenated, one olefinic), and four sp² quaternary carbons (Tables 2 and 3). The data displayed characteristic signals similar to those of xylaolide A.²⁰ Analysis of the 2D NMR spectra of **7** revealed that C-12 was substituted by a methoxy group in **7** instead of a hydroxy group in xylaolide A, which was supported by the HMBC correlation from the methoxy proton (δ_H 3.44) to C-12 (δ_C 66.2) (Fig. 2). Other parts of the structure, along with the relative configuration, were consistent to those of xylaolide A by analysis of the ROESY spectrum (Fig. 3). The above assignments accounted for a molecular formula of C₁₃H₁₈O₄, in accord with the HRESIMS result (*m/z* 261.1098 [M + Na]⁺, calcd for C₁₃H₁₈O₄Na, 261.1098). Therefore, compound **7** was named as 12-*O*-methyl-xylaolide A.

Compound **8**, a colourless oil, had the molecular formula C₁₅H₂₀O₂ determined by HREIMS analysis at *m/z* 232.1460 [M]⁺ (calcd C₁₅H₂₀O₂, 232.1463), requiring six degrees of unsaturation. The 1D NMR data presented signals for four methyl singlets (δ_H 1.37, 1.38, 1.40, 1.78; δ_C 27.6, 29.2, 29.4, 15.4), three methylenes, two sp³ quaternary carbons, and two pairs of α , β -unsaturated ketone groups (δ_H 6.64, δ_C 142.3, 135.4, 197.8; δ_H 5.93, δ_C 130.0, 190.4, 207.1) (Tables 2 and 3). The 1D NMR data accounted for four degrees of unsaturation, implying the existence of two rings in **8**. The planar structure of **8** was established by analysis of the 2D NMR data. The key HMBC correlations from H₃-14 (δ_H 1.37) to C-1 (δ_C 50.4), C-5 (δ_C 38.4), and C-6 (δ_C 43.2), and from H-1 (δ_H 2.87, 2.59) to C-2 (δ_C 197.8), and from H₃-15 (δ_H 1.78) to C-2, C-3 (δ_C 135.4), and C-4 (δ_C 142.3), along with the ¹H-¹H COSY correlation between H-4 (δ_H 6.64) and H-5 (δ_H 2.83, 2.50) (Fig. 2), allowed the completion of a six-

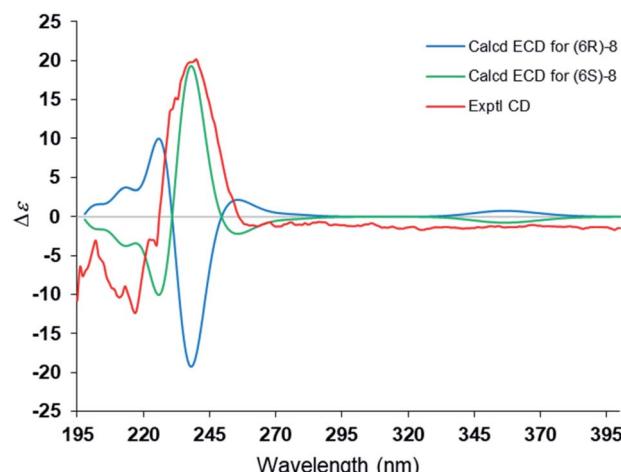


Fig. 6 Comparison of the experimental CD and calculated ECD spectra of compound **8**.

membered ring. Moreover, the characteristic HMBC correlations from H₃-12 (δ_H 1.38), H₃-13 (δ_H 1.40) to C-7 (δ_C 190.4), C-10 (δ_C 55.2), and C-11 (δ_C 45.1), and from H-8 (δ_H 5.93) to C-7 and C-9 (δ_C 207.1) (Fig. 2) enabled the accomplishment of a five-membered ring. Besides, the HMBC correlation from H₃-14 to C-7 suggested that the two rings are connected *via* C-6-C-7 (Fig. 2). Therefore, the planar structure of **8** was determined as shown in Fig. 1. Notably, compound **8** represents an unusual sesquiterpene skeleton in nature, holding structural similarity to that of the cuparane-type sesquiterpenes, and we herein designate the skeleton of **8** as 14(7 \rightarrow 6)-cuparane.

The steric hindrance between the five- and six-membered rings made it possible to establish the absolute configuration of C-6 by computational methods. The possible conformers of (6S)-**8** were generated by the MMFF94s force field conformation search, the geometries with population higher than 1% were further optimized by density functional theory method at the B3LYP/6-31G(d,p) level on Gaussian 09 program package²¹ to give eight predominant conformers within relative energies 3 kcal mol⁻¹. These eight conformers were subjected to theoretical calculation of ECD spectra by using the time-dependent DFT method at the B3LYP/6-31G(d,p) in air. As shown in Fig. 6, the calculated ECD spectra for (6S)-**8** displayed a similar curve with the experimental CD. Thus, the absolute configuration of **1** was determined as 6S. Compound **8** was trivially named as lingzhidienone.

Compound **9**, named ganotheaecolin A,²² a known isolate reported more recently was encountered in this study. Ganotheaecolin A represented a novel type of steroid with a unique naphtha[1,8-*e*]azulene ring system, and it was suggested that **9** exhibited cell differentiation-stimulating activity by a dose-dependent manner and reach a maximum effect at 10 μ M. With this fascinating molecule in hand, we evaluated the cytotoxicity of **9** and its acetylated product **9a** (5-*O*-acetyl ganotheaecolin (A)) against the five human cancer cell lines HL-60, MCF-7, SW480, A549, and SMMC-7721. Interestingly, compound **9** displayed weak cytotoxicity against five human

Table 3 ¹H NMR Data of Compounds **7** and **8** (600 MHz, CD₃OD)

| No. | 7 | 8 |
|------|---------------------------------------|---|
| 1 | | 2.87, d (15.8), 2.59, d (15.8) |
| 3 | 7.77, s | |
| 4 | | 6.64, br. dd (6.0, 3.0) |
| 5 | | 2.83, dd (17.6, 2.4), 2.50, dd (17.6, 2.4) |
| 6 | 2.59, overlapped | |
| 7 | 1.79, m, 1.71, overlapped | |
| 8 | 1.44, m | 5.93, s |
| 9 | 4.87, d (3.2) | |
| 10 | 1.70, overlapped, 1.42, overlapped | 2.36, s (2H) |
| 11 | 1.01, t (7.0) | |
| 12 | 4.32, d (12.4), 4.29, d (12.4) | 1.38 s |
| 13 | | 1.40, s |
| 14 | | 1.37, s |
| 15 | | 1.78, s |
| MeO- | 3.44, s | |



Table 4 Cytotoxicity of 9 toward five human cancer cell lines

| Sample | IC ₅₀ (μM) | HL-60 | A-549 | SMMC-7721 | MCF-7 | SW480 |
|--------|-----------------------|--------|--------|-----------|--------|-------|
| 9 | 18.8 | 32.3 | 25.1 | 18.1 | 22.3 | |
| DDP | 4.7 | 27.6 | 16.0 | 34.8 | 16.1 | |
| Taxol | <0.008 | <0.008 | <0.008 | <0.008 | <0.008 | |

cancer cell lines with IC₅₀ values of 18.0–32.3 μM (Table 4), while 9a was devoid of activity (IC₅₀ > 40 μM). The results suggested that the 5-OH of 9 was indispensable for its cytotoxicity.

The gymnomitrane and 14(7→6)-cuparane scaffolds appear to be biogenetically related. As shown in Scheme 1, the hypothetical biosynthetic pathways of the gymnomitrane (1–6, compound 2 as the illustrative example) and 14(7→6)-cuparane (8) are described. First of all, the farnesyl pyrophosphate (FPP) undergoes cascade 1,6- and 7,11-cyclization reactions and a 1,4-hydride shift from C-6 to C-10 to give the key intermediate cuparane skeleton. The cuparane yields the gymnomitrane and 14(7→6)-cuparane skeleton via two ways. One way is the synchronous methyl and hydride migrations to produce the 14(7→6)-cuparane scaffold. The other way is two methyl migrations, *i.e.* 14(7→6) and 13(11→7), in combination with a 2,11-cyclization reaction to produce the gymnomitrane scaffold. Further enzymatic oxidations and dehydration reactions yield 2 and 8.

All the compounds except 6 and 7 were evaluated for their inhibitory activity against NO production. However, none of

them showed remarkable inhibitory activities of NO production in murine monocytic RAW 264.7 macrophages at the concentration of 25 μM.

Compounds 1–5, 8, 9, and 9a were screened for their cytotoxicity against five human cancer cell lines (HL-60, A-549, SMMC-7721, MCF-7, SW480). Compounds 1–5, 8, and 9a were inactive in the cytotoxicity assay (IC₅₀ > 40 μM). Only compound 9 displayed inhibitory activity against the five human cancer cell lines with IC₅₀ values ranging from 18.0 to 32.3 μM (Table 4), indicating that 5-OH is essential for its activity.

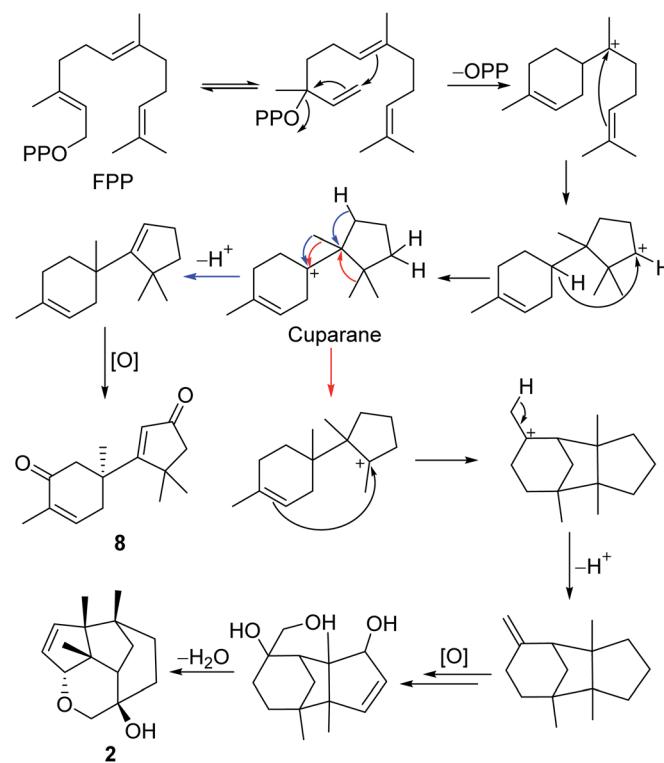
Conclusions

In summary, chemical investigation on the edible and medicinal mushroom *Ganoderma lingzhi* afforded six new gymnomitrane-type sesquiterpenoids (1–6), an unusual sesquiterpenoid (8), and a new polyketide (7) as well as a known steroid (9). The absolute configurations of the isolates were unambiguously determined *via* single crystal X-ray diffraction analysis, and ECD calculation. The biosynthetic pathway of 1–6 and 8 were also discussed. Among the structures, 2 is a tetracyclic boat-like rigid molecule with a 10,15-epoxy group. Compound 8 possessed an unusual 14(7→6)-cuparane scaffold. It is noteworthy that the gymnomitrane-type sesquiterpenoids have rarely been encountered from fungal origin^{6,14,23} and this work represents the first report of clustered gymnomitrane from *Ganoderma*, or even from fungi. This work also provides evidence consistent with the genome sequence data of *Ganoderma*. Although no promising biological assay results are reported in this study, this does not mean that the sesquiterpenoids should be neglected in the development of quality criteria for *Ganoderma*. More work on identifying the constituents of *Ganoderma* should be put into practice in the course of preclinical studies prior to clinical use.

Experimental section

General experimental procedures

Optical rotations were measured by a JASCO P-1020 digital polarimeter (Horiba, Kyoto, Japan). A UV-2401PC UV-visible recording spectrophotometer (Shimadzu, Kyoto, Japan) was used to record the ultraviolet (UV) spectra. A Chirascan circular dichroism spectrometer (Applied Photophysics Limited, Leatherhead, Surrey, UK) was used to record the CD spectra. 1D and 2D NMR spectra were obtained on Bruker Avance III 600 MHz or Ascend 800 MHz spectrometers (Bruker Corporation, Karlsruhe, Germany). An Agilent 6200 Q-TOF MS system (Agilent Technologies, Santa Clara, CA) was used to acquire the HRESIMS data. A Waters AutoSpec Premier P776 MS system (Waters Corporation, Milford, MA) was used to acquire the HREIMS data. An APEX II DUO spectrophotometer (Bruker AXS GmbH, Karlsruhe, Germany) was applied for performing the single crystal X-ray diffraction experiments. Column chromatography (CC) were run on Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) and silica gel (Qingdao Haiyang Chemical Co., Ltd, Qingdao, China). A Büchi Sepacore System (pump manager C-615, pump modules C-605, and fraction



Scheme 1 Proposed biosynthetic pathway of 2 and 8.



collector C-660) (Büchi Labortechnik AG, Flawil, Switzerland) was used to perform medium pressure liquid chromatography (MPLC), equipped with a column (400 mm \times 7.4 mm i.d., 40–75 μm , flow rate 40 mL min $^{-1}$) filled with Chromatorex C-18 (Fuji Silysia Chemical Ltd., Kasugai, Japan) RP-C18 silica gel. An Agilent 1260 liquid chromatography system (Agilent) equipped with an ODS column (Zorbax SB-C18, 150 mm \times 9.4 mm i.d., 5 μm , flow rate 10 mL min $^{-1}$) was used for preparative high performance liquid chromatography (prep-HPLC).

Fungal material

The fruiting bodies of *G. lingzhi* were purchased from the herbal trading market of Kunming Luoswan International Trade City in 2015, and identified by Prof. Yu-Cheng Dai (Beijing Forestry University), an expert in the field of mushroom taxonomy. A voucher specimen (No. HFC 20150518) of *G. lingzhi* was deposited in the Herbarium of Ethnic Medicinal Plants of South-Central University for Nationalities (SCUEC).

Extraction and isolation

The air-dried and powdered fruiting bodies of *G. lingzhi* (10.0 kg) was extracted with 95% ethanol three times (three days each). The extract was evaporated under reduced pressure and partitioned between ethyl acetate and water four times to give a crude extract (400 g). The crude extract was subject to silica gels with a stepwise gradient of petroleum ether/acetone (from 20 : 1 \rightarrow 1 : 1, v/v, totally 20 L) to afford five fractions (A–E).

Fraction B (45 g) was subjected to MPLC with a solvent gradient system of MeOH/H₂O (from 80 : 20 \rightarrow 100 : 0, v/v, 35 mL min $^{-1}$) to obtain twenty-two subfractions (B1 to B22). Subfraction B16 (40 mg) was further purified by Sephadex LH-20 (MeOH) and then B16d was separated by prep-HPLC using a gradient elution (MeCN/H₂O, 30 : 70 \rightarrow 50 : 50, v/v, 30 min, 7 mL min $^{-1}$) to yield compound 2 (0.7 mg, $t_{\text{R}} = 8.5$ min). Subfraction B1 (22 mg) was applied to Sephadex LH-20 (MeOH) to afford four minor fractions (B1a to B1d). Compound 8 (2.2 mg, MeCN/H₂O, 10 : 90 \rightarrow 35 : 65, v/v, 30 min, 7 mL min $^{-1}$, $t_{\text{R}} = 23.2$ min) was obtained from subfraction B1c via prep-HPLC. Subfraction B17 (65 mg) was further purified by prep-HPLC using a gradient elution (MeCN/H₂O, 60 : 40 \rightarrow 90 : 10, 30 min, 7 mL min $^{-1}$) to yield compound 9 (32 mg, $t_{\text{R}} = 27.2$ min).

Fraction C (27 g) was subjected to MPLC with a gradient solvent system of MeOH/H₂O (80 : 20 \rightarrow 100 : 0, v/v, 35 mL min $^{-1}$) to obtain twenty-two subfractions (C1 to C15). Subfraction C2 (45 mg) was further purified by Sephadex LH-20 (MeOH), then fractions C2e, C2f and C2g were separated by prep-HPLC to afford compound 1 (0.9 mg, MeCN/H₂O, 27 : 73 \rightarrow 42 : 58, v/v, 20 min, 7 mL min $^{-1}$, $t_{\text{R}} = 13.5$ min), compounds 6/7 mixture (2.6 mg, MeCN/H₂O, 15 : 85 \rightarrow 30 : 70, v/v, 20 min, 7 mL min $^{-1}$, $t_{\text{R}} = 16.0$ min), and compound 3 (1.1 mg, MeCN/H₂O, 20 : 80 \rightarrow 40 : 60, 20 min, 7 mL min $^{-1}$, $t_{\text{R}} = 17.2$ min), respectively. Subfraction C3 (105 mg) was separated by Sephadex LH-20 (MeOH) yielding six minor fractions (C3a to C3f). Next, fraction C3e was applied on Sephadex LH-20 (Acetone) to afford five minor fractions (C3e1 to C3e5), then C3e4 were treated by prep-HPLC to afford compounds 4 (5.0 mg, MeCN/

H₂O, 20 : 80 \rightarrow 40 : 60, v/v, 7 mL min $^{-1}$, 20 min, $t_{\text{R}} = 13.5$ min), 5 (10.0 mg, MeCN/H₂O, 20 : 80 \rightarrow 40 : 60, 20 min, 7 mL min $^{-1}$, $t_{\text{R}} = 4.5$ min), respectively.

10 α -Hydroxy-gymnomitr-3-en-15-al (1). Colourless needles; m.p. 157.9–160.4 °C; $[\alpha]_{\text{D}}^{23} +6.44$ (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ϵ): 241.60 (3.23); ¹H NMR data (Table 1); ¹³C NMR data (Table 2); HREIMS *m/z* 234.1602 [M]⁺ (calcd for C₁₅H₂₂O₂, 234.1620).

Single crystal X-ray diffraction data for 1. Crystal data for Cu_1_0m: C₁₅H₂₂O₂, *M* = 234.32, *a* = 7.70800(10) Å, *b* = 12.5490(2) Å, *c* = 13.1278(2) Å, α = 90°, β = 90°, γ = 90°, *V* = 1269.82(3) Å³, *T* = 100(2) K, space group P2₁2₁2₁, *Z* = 4, $\mu(\text{CuK}\alpha)$ = 0.621 mm $^{-1}$, 8045 reflections measured, 2306 independent reflections ($R_{\text{int}} = 0.0442$). The final R_1 values were 0.0513 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.1314 ($I > 2\sigma(I)$). The final R_1 values were 0.0513 (all data). The final $wR(F^2)$ values were 0.1315 (all data). The goodness of fit on F^2 was 1.061. Flack parameter = 0.09(6). The crystallographic data were deposited to the Cambridge Crystallographic Data Centre (CCDC) with the No. CCDC 1561836. Copies of the data are available for free from Cambridge Crystallographic Data Centre.

10 α ,15-Epoxy-gymnomitr-8-en-3 β -ol (2). Colourless needles; m.p. 172–175 °C; $[\alpha]_{\text{D}}^{22} -47.67$ (*c* 0.10, MeOH); ¹H NMR data (Table 1); ¹³C NMR data (Table 2); HREIMS *m/z* 234.1617 [M]⁺ (calcd for C₁₅H₂₂O₂, 234.1620).

15-Hydroxy-gymnomitr-3-en-5-one (3). White amorphous powder; $[\alpha]_{\text{D}}^{23} +174.74$ (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ϵ): 244.20 (3.87); ¹H NMR data (Table 1); ¹³C NMR data (Table 2); HRESIMS *m/z* 257.1514 [M + Na]⁺ (calcd for C₁₅H₂₂O₂Na, 257.1512).

Gymnomitr-3-ene-13,15-dioic acid (4). Colourless oil, $[\alpha]_{\text{D}}^{23} +151.33$ (*c* 0.09, MeOH); ¹H NMR data (Table 1); ¹³C NMR data (Table 2); HRESIMS *m/z* 263.1291 [M – H][–] (calcd for C₁₅H₁₉O₄, 263.1289).

Methyl 5 β ,8 β -dihydroxy-gymnomitr-3-en-15-oate (5). Colourless needles; m.p. 137.5–142.2 °C; $[\alpha]_{\text{D}}^{24} +86.19$ (*c* 0.14, MeOH); UV (MeOH) λ_{max} (log ϵ): 221.50 (3.57); ¹H NMR data (Table 1); ¹³C NMR data (Table 2); HRESIMS *m/z* 303.1565 [M + Na]⁺ (calcd for C₁₆H₂₄O₄Na, 303.1567).

Single crystal X-ray diffraction data for 5. Crystal data for Cu_5_0m: C₁₆H₂₄O₄, *M* = 280.35, *a* = 6.4948(2) Å, *b* = 7.4524(3) Å, *c* = 15.2812(6) Å, α = 90°, β = 101.0040(10)°, γ = 90°, *V* = 726.04(5) Å³, *T* = 100(2) K, space group P2₁, *Z* = 2, $\mu(\text{CuK}\alpha)$ = 0.736 mm $^{-1}$, 7540 reflections measured, 2510 independent reflections ($R_{\text{int}} = 0.0263$). The final R_1 values were 0.0327 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.0844 ($I > 2\sigma(I)$). The final R_1 values were 0.0327 (all data). The final $wR(F^2)$ values were 0.0845 (all data). The goodness of fit on F^2 was 1.056. Flack parameter = 0.17(3). The crystallographic data were submitted to the Cambridge Crystallographic Data Centre (CCDC) with the No. CCDC 1851594. These data can be accessed free from Cambridge Crystallographic Data Centre.

3 β ,4 β -Dihydroxy-gymnomitr-9-one (6). Colourless oil; ¹H NMR data (Table 1); ¹³C NMR data (Table 2); HREIMS *m/z* 252.1724 [M]⁺ (calcd for C₁₅H₂₂O₃, 252.1725).

12-O-Methyl-xylolide A (7). Colourless oil; ¹H NMR data (Table 3); ¹³C NMR data (Table 2); HRESIMS *m/z* 261.1098 [M + Na]⁺ (calcd for C₁₃H₁₈O₄Na, 261.1097).



Lingzhidieneone (8). Colourless oil; $[\alpha]_D^{23} +22.31$ (*c* 0.13, MeOH); UV (MeOH) λ_{max} ($\log \epsilon$): 230.80 (4.23); ^1H NMR data (Table 3); ^{13}C NMR data (Table 2); HREIMS m/z 232.1460 [M] $^+$ (calcd for $\text{C}_{15}\text{H}_{20}\text{O}_2$, 232.1463); CD (MeOH) λ_{max} ($\Delta \epsilon$): 217 (−12.3), 240 (+20.2), 260 (−1.2).

Preparation of 5-O-acetyl-ganotheaecolin A (9a). To a solution of ganotheaecolin A (2.0 mg) in anhydrous pyridine (0.2 mL) was added Ac_2O (0.5 mL), and the mixture was stirred at 25 °C until the reaction was complete (judged by the disappearance of the starting material by TLC). Excess ethanol was added to the reaction mixture and evaporated under reduced pressure. The crude product was purified by prep-HPLC (MeCN/H₂O, 70 : 30 → 90 : 10, 25 min, 7 mL min^{−1}) to yield the corresponding 5-O-acetyl derivative. Application of this procedure afforded the new acetate 5-O-acetyl-ganotheaecolin A (9a).

5-O-Acetyl-ganotheaecolin A (9a): ^1H NMR (500 MHz, CDCl_3): H-1 (δ_{H} 2.59, br. d, *J* = 18.8 Hz; δ_{H} 1.75, overlapped), H-2 (δ_{H} 6.18, ddd, *J* = 10.0, 5.0, 2.5 Hz); H-3 (δ_{H} 6.35, dd, *J* = 10.0, 2.5 Hz); H-11 (δ_{H} 2.31, ddd, *J* = 15.0, 15.0, 3.0; δ_{H} 1.01, ddd, *J* = 15.0, 4.7, 2.5); H-12 (δ_{H} 1.96, ddd, *J* = 15.0, 5.0, 3.0 Hz; δ_{H} 1.34, br. d, *J* = 15.0 Hz); H-14 (δ_{H} 3.41, dd, *J* = 12.5, 7.2 Hz); H-15 (δ_{H} 1.79, overlapped; δ_{H} 1.61, overlapped); H-16 (δ_{H} 1.74, overlapped; δ_{H} 1.41, overlapped); H-17 (δ_{H} 1.61, m); H-18 (δ_{H} 0.79, s, 3H); H-19 (δ_{H} 0.72, s, 3H); H-20 (δ_{H} 2.14, m); H-21 (δ_{H} 1.04, d, *J* = 6.7 Hz); H-22 (δ_{H} 5.20, dd, *J* = 15.3, 7.5 Hz); H-23 (δ_{H} 5.24, dd, *J* = 15.3, 7.0 Hz); H-24 (δ_{H} 1.86, m); H-25 (δ_{H} 1.47, m); H-26 (δ_{H} 0.82, d, *J* = 6.8 Hz); H-27 (δ_{H} 0.84, d, *J* = 6.8 Hz); H-28 (δ_{H} 0.92, d, *J* = 6.8 Hz); ^{13}C NMR (125 MHz, CDCl_3): C-1 (δ_{C} 40.0); C-2 (δ_{C} 137.3); C-3 (δ_{C} 127.0); C-4 (δ_{C} 168.9); C-5 (δ_{C} 131.9); C-6 (δ_{C} 184.5); C-7 (δ_{C} 127.0); C-8 (δ_{C} 162.3); C-9 (δ_{C} 74.2); C-10 (δ_{C} 41.4); C-11 (δ_{C} 33.1); C-12 (δ_{C} 33.8); C-13 (δ_{C} 43.5); C-14 (δ_{C} 47.8); C-15 (δ_{C} 27.0); C-16 (δ_{C} 27.2); C-17 (δ_{C} 56.7); C-18 (δ_{C} 18.7); C-19 (δ_{C} 19.8); C-20 (δ_{C} 40.5); C-21 (δ_{C} 22.0); C-22 (δ_{C} 134.9); C-23 (δ_{C} 133.0); C-24 (δ_{C} 43.5); C-25 (δ_{C} 33.2); C-26 (δ_{C} 19.8); C-27 (δ_{C} 20.2); C-28 (δ_{C} 17.8).

Bioassays

Nitric oxide production in RAW 264.7 macrophages

The RPMI 1640 medium (Hyclone, Logan, UT) containing 10% FBS was used to culture the murine monocytic RAW 264.7 macrophages. The compounds were dissolved in DMSO and further diluted in medium to produce different concentrations. The culture medium and cell mixture were dispensed into 96-well plates (2×10^5 cells per well) and maintained at 37 °C under 5% CO₂ in a humidified atmosphere. After preincubation for 24 h, serial dilutions of the test compounds were added into the cells, up to the maximum concentration 25 μM , then LPS was added to a concentration 1 $\mu\text{g mL}^{-1}$ and incubation continued for 18 h. After addition of 100 μL of Griess reagent (reagent A and reagent B, Sigma, St. Louis, Mo) to 100 μL of each supernatant from the LPS-treated or LPS- and compound-treated cells in triplicate and incubation for 5 min, NO production of each cell was assessed by sample absorbance at 570 nm by a 2104 Envision Multilabel Plate Reader. L-N^G-Monomethyl arginine (L-NMMA) was used as a positive control.

Cytotoxicity against five human cancer cell lines. The following five human cancer cell lines were used: the HL-60

(ATCC CCL-240) human myeloid leukemia; SMMC-7721 human hepatocellular carcinoma; A-549 (ATCC CCL-185) lung cancer; MCF-7 (ATCC HTB-22) breast cancer; SW-480 (ATCC CCL-228) human colon cancer. The cell line SMMC-7721 was bought from China Infrastructure of Cell Line Resources (Beijing, China), and others were bought from American Type Culture Collection (ATCC, Manassas, VA). All cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Hyclone) and maintained at 37 °C under 5% CO₂ in a humidified atmosphere. Colorimetric measurements of the amount of insoluble formazan which was produced in living cells based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) was used to assess cell viability. In brief, each well of a 96-well cell culture plate was seeded with 100 μL of adherent cells and kept for 12 h for adherence, and then added with test compounds, however, suspended cells were seeded before added with test compounds with both the same density of 1×10^5 cells per mL every 100 μL of culture medium. After different concentrations of test compounds addition, each cancer cell line was incubated for 48 h in triplicate. Cisplatin was used as positive control. After the incubation, each well was treated with MTT (100 μg) and incubation continued for 4 h at 37 °C. After removal of the 100 μL culture medium, the cells were lysed with 20% SDS-50% DMF (100 μL). The remaining lysates were subjected to measure of optical density at 595 nm with a 96-well microtiter plate reader. The IC₅₀ value for each compound was calculated by a published method.²⁴

Quantum chemistry calculation details

A conformation search based on molecular mechanics with MMFF94s force fields were performed for (6*S*)-8 gave 10 stable conformers with distributions higher than 1%.^{25,26} All these conformers were further optimized by the density functional theory method at the B3LYP/6-31G(d,p) level in Gaussian 09 program package,²¹ leading to six ((6*S*)-8a–(6*S*)-8f) conformers within 3 kcal mol^{−1} energy threshold from global minimum, respectively. The predominant conformers were subjected to theoretical calculation of ECD using time-dependent density functional theory (TDDFT) at B3LYP/6-31G(d,p) level with IEFPCM model in air based on B3LYP/6-31G(d,p) optimized conformers. The calculated ECD curves for (6*S*)-8 were weighted using SpecDis 1.71 with σ = 0.2 eV, and UV shift 11 nm, respectively.²⁷ The ECD curve of the enantiomer (6*R*)-8 was generated by SpecDis by the function “enantiomeric ECD”.

Conflicts of interest

There are no conflicts to declare.

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