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# Clavicorolides A and B, Sesquiterpenoids from the Fermentation Products of Edible Fungus *Clavicorona pyxidata*

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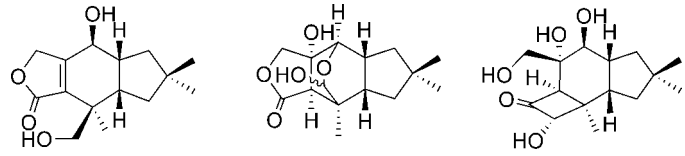
## ABSTRACT




**Clavicorolides A (1) and B (2), two sesquiterpenoids possessing a novel backbone named as Clavicoronane-type, together with one new and two known Protoilludane-type sesquiterpenoids, namely, Tsugicolines M (3), A (4), and C (5), and Sterpurane (6), and Lactarane-type sesquiterpenoid Lactarorufin A (7), were isolated from the fermentation products of *Clavicorona pyxidata* YB2005. Their structures including relative and absolute configurations were elucidated on the basis of NMR data and analysis of X-ray single crystal diffraction.**

Mushrooms have been known to be a rich source of natural products.[1−4] *Clavicorona pyxidata* is a wild edible mushroom that is used in traditional medicine in China. The fruiting bodies of this species have been investigated for bioactive natural products, which resulted in the isolation of various types of compounds including clavicoronic acid, a novel inhibitor of reverse transcriptases.[5] Because of the limitation of obtaining wild mushrooms, which usually replicate in small quantity and a very short season annually, we turned to the sustainable mycelia fermentation approach to mining macrofungal natural products. The strain *C. pyxidata* YB2005 was isolated from the wild fruiting body and identified by ITS methods. It was cultured on PDA media (9 L) in Petri dishes for 25 days at 25 °C and extracted with EtOAc/MeOH/AcOH (80:15:5, v/v/v) to afford a brown crude extract that was partitioned between water and EtOAc. The organic layer was subjected to column chromatography over RP-18 Si gel, Sephadex LH-20, and normal phase Si gel successively to afford two novel sesquiterpenoids, namely, Clavicorolides A (**1**) and B (**2**) assigned as Clavicoronane-type. These compounds were obtained together with five more sesqiuterpenoids including one new and two known Protoilludane-type sesquiterpenoids, Tsugicolines M (**3**), A (**4**), and C (**5**), and Sterpurane (**6**) and Lactarane-type sesquiterpenoid Lactarorufin A (**7**) (Figure 1). This report describes the structural characterization and elucidation of Clavicorolides A (**1**) and B (**2**) and Tsugicolines M (**3**) and discusses a putative biosynthetic pathway from FPP to Clavicoronane via Protoilludane or Marasmane (Scheme 1).

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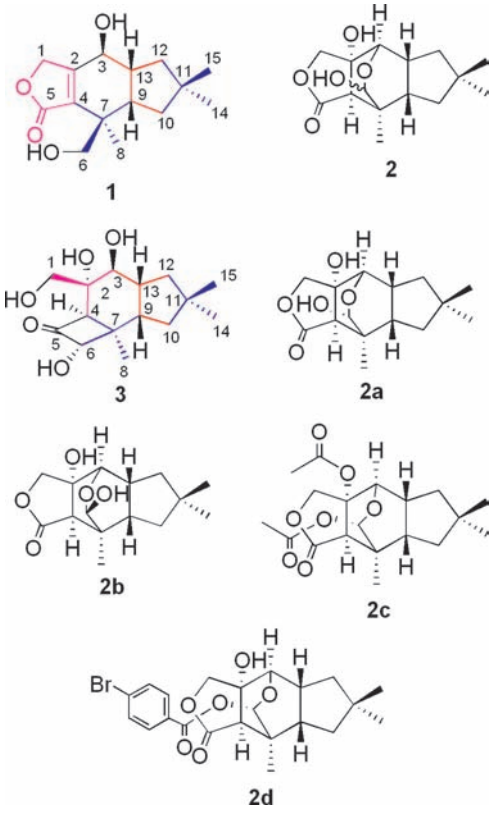


**Figure 1.** *Claviorona pyxidata* metabolites: Clavicorolides A (**1**) and B (**2a**/**2b**), Tsugicolines M (**3**), 2,6-*O*-diacetyl Clavicorolide B (**2c**), and 6-*O*-*p*-bromobenzoyl Clavicorolide B (**2d**).

Clavicorolide A (**1**) was isolated as a colorless crystal (from aqueous acetone), mp 163.1 °C, $[\alpha]^{20}_{D}$ −1.4 (*c* 0.38, MeOH). The molecular formula was determined to be $C_{15}H_{22}O_4$ according to the HR Q-TOF MS data (*m*/*z* 267.1565 $[M + H]^+$ and 289.1395 $[M + Na]^+$). The IR spectra showed the absorptions for hydroxyl (3285 $cm^{-1}$), ester carbonyl (1741 $cm^{-1}$), and unsaturated double bond (1649 $cm^{-1}$) groups. Inspection of the $^1H$ and $^{13}C$ NMR spectra (Table S1, Supporting Information) revealed the presence of three tertiary methyl groups (three singlets at $\delta_H$ 1.04, 1.11, and 1.13), four methylenes, two methines, and five quaternary carbons, including two oxymethylenes, one oxymethine, and one carbonyl group that attributed to four oxygen substitutions in **1**. The $^1H$ NMR and HSQC spectra indicated the presence of two hydroxyl groups ($\delta_H$ 1.04, d, 7.9; $\delta_H$ 3.88, t, 5.6), which was confirmed by the acetylation of **1** in $Ac_2O$/pyridine (1:1) for overnight at room temperature to yield the diacetyl product having a quasi-molecular ion peak at *m*/*z* 373.2 $[M + Na]^+$ in ESI-MS. Moreover, the coupling patterns of these two hydroxyl protons of **1** (Table S1, Supporting Information), one doublet and one triplet, suggested the presence of one hydroxymethine and one hydroxymethyl group, which were assigned to C-3 and C-6, respectively, on the basis of the HMBC correlations. The protons of another oxymethylene (C-1) at $\delta_H$ 4.82 and 4.77 showed HMBC correlations to three quaternary carbons at $\delta_C$ 164.3 (C-2), 127.8 (C-4), and 172.4 (C-5), respectively, indicating the presence of an α,*β*-unsaturated *γ*-lactone moiety (Figure 1, in pink). The HMBC correlations from the protons of the three methyl groups at $\delta_H$ 1.04 (s, H-14), 1.11 (s, H-15) and 1.13 (s, H-8) and that from the protons of the hydroxymethyl group (C-6) at $\delta_H$ 3.47 and 3.56 to corresponding carbons determined two five-carbon moieties (Figure 1, in blue). The five-carbon moiety including C-4 was composed of one hydroxymethyl, one methyl, one methine and another quaternary carbon, indicating that both the hydroxymethyl and methyl groups were located at the *gem*-position, the *β*-position of the lactone carbonyl group. Another five-carbon moiety was composed of two methyls, two methylenes and one quaternary carbon, indicating the presence of *gem*-dimethyl groups. The $^1H-^1H$ COSY correlations determined the structure of one more five-carbon moiety including C-3, C-9, C-10, C-12, and C-13 (Figure 1, in red). The connection between C-2 and C-3 was assigned on the basis of the HMBC correlations between H-3 and C-2, even though the common three-bond $^1H-^{13}C$ long-range correlations between H-1 and C-3, and H-3 and C-1 were not observed. On the basis of the above, a fused α,*β*-unsaturated *γ*-lactone, cyclohexane, and cyclopentane ring system was assigned to **1**, which represents a new type of sesquiterpene backbone, named as Clavicoronane-type.[6]

The relative configuration of **1** was determined by NOESY and X-ray single crystal diffraction experiments. The presence of NOEs between H-9 and H-13, and H-9 and $H_3$-15 indicated the *β*-orientation of these protons and $CH_3$-15. Further, the NOESY cross-peaks between H-3 and $H_3$-8 established the α-orientation of H-3 and $CH_3$-8. Finally, the relative configuration of **1** was fully determined by X-ray diffraction analysis of a single crystal obtained from aqueous acetone (Figure 2).[7]

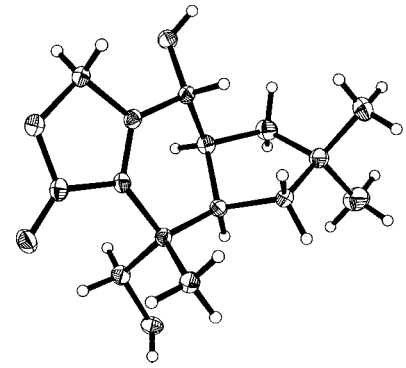

**Figure 2.** X-ray structure of Clavicorolide A (**1**).

Although naturally isolated, compound **2** was pure as indicated by rigorous TLC detections using several developing solvent systems. The HR Q-TOF MS showed the quasi-molecular ion peaks at *m*/*z* 283.1647 $[M + H]^+$ and 305.1470 $[M + Na]^+$; however, the $^1H$ and $^{13}C$ NMR spectra indicated

(6) *Dictionary of Natural Products*; Taylor & Francis Group, http://dnp.chemnetbase.com, accessed 2008.

that **2** would be a mixture of two compounds with the ratio of ca. 1:0.6 (**2a**:**2b**) revealed by the proton signal integrations (Table S2, Supporting Information). Comprehensive inspection of the NMR data ($^1$H, $^{13}$C, HSQC, HMBC and NOE) revealed that **2a** and **2b**, similar to **1**, have a backbone of a fused ring system of a $\gamma$-lactone, cyclohexane, and cyclopentane, but the lactone moiety in **2a**/**2b** was saturated, and C-2 was oxygen-substituted. Another difference between **1** and **2a**/**2a** is that C-6 was oxymethine in **2a**/**2b** rather than oxymethylene as in **1**. Moreover, the HMBC correlations showed an oxygen bridge between C-3 and C-6, indicating the presence of a hemiacetal at C-6 (Figure 1). Therefore, we speculated that **2a** and **2b** might be a pair of C-6 epimers. To confirm our speculation, we tried to separate the two

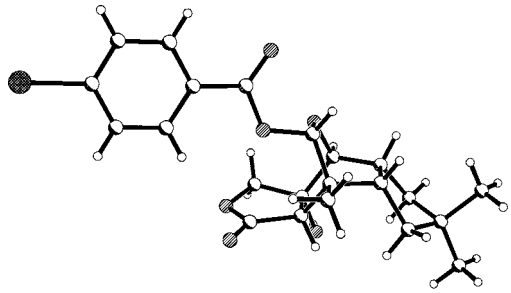

**Figure 3.** X-ray structure of 6-*O*-*p*-bromobenzoyl Clavicorolide B (**2d**).

epimers by acetylating the hemiacetal hydroxyl group first. The mixture of **2a** and **2b** was reacted with acetic anhydride in pyridine at room temperature. Surprisingly, only one product, **2c**, was obtained as colorless crystals with mp 138.5 °C and $[\alpha]^{20}_{D}$ +122.0 (*c* 0.05, $CHCl_3$).[8] The HR Q-TOF MS presented the quasi-molecular ion peak at *m*/*z* 389.1238 [M + Na]$^+$, indicating that two acetyl groups were introduced by acetylation. The assignments of the NMR data ($^1$H, $^{13}$C, HSQC, HMBC and NOE) (Table S3, Supporting Information) of **2c** revealed that both of the hydroxyl groups at C-2 and C-6 were acetylated and determined the planar structures of **2a**/**2b**. However, the relative configuration of C-6 was left to be resolved so that we could not definitely assign the structures of **2a**/**2b**. Accordingly, we performed a *p*-bromobenzoylation of Clavicorolide B (**2a** or **2b**) at the hydroxy group of C-6 to determine the absolute configuration by the analysis of X-ray single crystal diffraction. After **2a**/**2b** reacted with *p*-bromobenzoyl chloride,[9] the 6-*O*-*p*-bromobenzoylated product (**2d**) was obtained, which produced a single crystal in aqueous methanol at 4 °C. Hence, based on the X-ray diffraction analysis,[10] compound **2d** (Table S4, Supporting Information) was determined to be (2*R*,3*S*,4*S*,6*R*,9*S*,13*R*)-6-*O*-*p*-bromobenzoyl Clavicorolide B (Figures 1 and 3). Accordingly, the structures of compounds **2a**, **2b**, and **2c** were determined by NMR comparison with **2d**.

Tsugicoline M (**3**) was isolated as colorless oil, $[\alpha]^{20}_{D}$ −1.1 (*c* 1.3, MeOH). The IR spectrum (MeOH) exhibited absorptions for hydroxyl (3380 cm$^{-1}$), methyl and methylene groups (2950, 2867 cm$^{-1}$), and carbonyl (1770 cm$^{-1}$). The mass spectrum presented strong quasi-molecular ion peaks at *m*/*z* 285.1896 [M + H]$^+$ and *m*/*z* 307.1737 [M + Na$^+$]. The $^{13}$C NMR spectra (Table S5, Supporting Information) of **3** revealed the presence of 15 resonance signals attributed to one ketone, three methyls, three methylenes, five methines (two of them oxygen-bearing), and three sp$^3$ quaternary carbons (one of them oxygen-bearing), indicating a three-ring sesquiterpene backbone. Following the similar strategy above to interpret the NMR spectra of **3** (Table S5, Supporting Information), two five-carbon moieties (Figure 1, in blue) were determined on the basis of the HMBC correlations from the protons of three methyl groups at $\delta_H$ 0.94 (s, H-8), 1.06 (s, H-15), and 1.15 (s, H-14) to the corresponding carbons, indicating the presence of *gem*-dimethyl groups, the same as in compound **1**. Moreover, the $^1$H−$^1$H COSY correlations were used to determine the structure of one more five-carbon moiety including C-3, C-9, C-10, C-12, and C-13 (Figure 1, in red). Additionally, a four-carbon moiety was recognized by analyzing the HMBC correlations of the hydroxymethyl protons (H-1) to the corresponding carbons (C-2, C-3, C-4) (Figure 1, in pink). The assignment of the ketone to C-5 was based on the HMBC correlations of H-4 and H-6 to $\delta_C$ 208.2. Thus, a Protoilludane-type backbone, characterized by a fused cyclobutanone, cyclohexane, and cyclopentane ring system, was assigned to **3**, the same as other Tsugicolines,[11] and compound **3** was named Tsugicoline M. The NOEs between H-6 and H-9, H-6 and H-13, H-9 and H-13, H-9 and $H_2$-1, and H-9 and $H_3$-15 indicated the $\beta$-orientation of these protons and $HOCH_2$-1 and $CH_3$-15. Furthermore, the NOESY cross-peaks between H-3 and H-4, and H-4 and $H_3$-8 indicated the α-orientation of these protons and $CH_3$-8. Therefore, the relative configuration of **3** was established (Figure 1).

(7) Crystallographic data for **1**: $C_{15}H_{22}O_4$; $M$ = 266.2; orthorhombic, $a$ = 6.4397(2), $b$ = 15.3604(4), $c$ = 31.2247(7) Å, space group $P2_12_12_1$, $Z$ = 8, $D_\chi$ = 1.223 Mg m$^{-3}$, $\mu$ = 0.09 $^{-1}$, $F$(000) = 1232; colorless prismatic crystals, dimension 0.4 × 0.4 × 0.7 mm$^3$. CCDC-683154 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Center, 12 Union Road, Canbridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

(8) The powder of 10 mg Clavicorolide B (**2**) was dissolved in 1 mL of $Ac_2O$/pyridine (1:1) and reacted for overnight at room temperature.

(9) A solution of Clavicorolide B (**2**) (5 mg) and 4-dimethylaminopyridine (1 mg) in 200 $\mu$L of anhydrous dichloromethane was stirred at 0 °C (in ice−water), and then the solution of *p*-bromobenzoyl chloride (9 mg) in 200 $\mu$L of anhydrous dichloromethane together with ca. 20 $\mu$L triethylamine were added dropwise. The mixture was stirred for 1 h at 0 °C (in ice−water) and quenched with cold brine.

(10) Crystallographic data for **2d**: $C_{22}H_{25}BrO_6$; $M$ = 465.3; orthorhombic, $a$ = 7.649(18), $b$ = 11.685(3), $c$ = 23.902(7) Å, space group $P2_12_12_1$, $Z$ = 4, $D_\chi$ = 1.447 Mg m$^{-3}$, $\mu$ = 1.959 mm$^{-1}$, $F$(000) = 956; colorless prismatic crystals, dimension 0.22 × 0.20 × 0.4 mm$^3$. CCDC-698197 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Center, 12 Union Road, Canbridge CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

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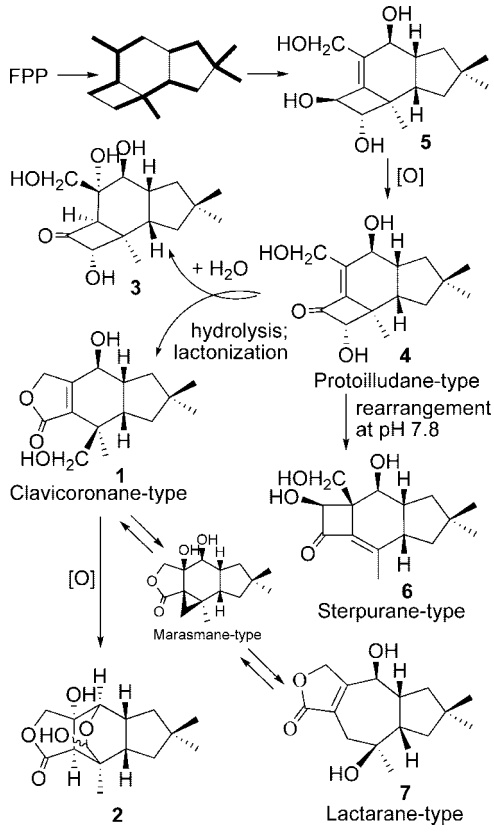
**Scheme 1.** Proposed Biogenetic Pathway of Clavicorolides A and B and Metabolites from *Clavicorona pyxidata* YB2005




Compounds **1**−**3** showed no evident antimicrobial and antitumor activities.[12]

Sesquiterpenoids are the largest family of natural products and have shown a variety of pharmacological potential, including cytotoxic,[13] anti-MRSA,[14] anti-HIV,[15] antiplasmodial,[16] and anti-inflammatory[17] biological activities. Macrofungi have been found to be a rich source of sesquiterpenoids with diverse backbones, such as protoilludane,[11] drimane,[18] dictyophorines,[19] and marasmic acid.[20] This is also illustrated by the isolation of four structure types of sesquiterpenoids from the fermentation products of *C. pyxidata* YB2005 in this study. Additionally, the isolation of two novel backbone sesquiterpenoids, **1** and **2**, from the strain *C. pyxidata* YB2005 was not very surprising given the fact that Protoilludane-type sesquiterpenoids such as compound **4**, which is the main components of this strain, may be converted to compound **1** via a putative oxidative C−C bond cleavage and lactonization reaction (Scheme 1). The mechanism of this oxidative C−C bond cleavage was unknown; however, a C−C bond hydrolysis was assumably involved (Figure S1, Supporting Information).[21] This C−C bond cleavage is very interesting because this type of reaction has not been reported in the biosynthesis of natural products. Moreover, the results of this study imply that post-terpene synthase (PTS) modifications including oxidation, rearrangement, C−C bond hydrolysis, and lactonization contribute to the structural diversities of sesquiterpenoids. Particularly, PTS modifications may be common in sesquiterpenoid backbone derivation, e.g., Lactarane-type (**7**) could be derived from Clavicoronane-type (**1**) via Marasmane-type or vice versa (Scheme 1), since the Marasmane-type sesquiterpenoid in Scheme 1 was isolated from mushrooms *Lactarius vellereus* and *Russula fetens* as well.[20] Alternatively, the backbone transformation of Lactarane to Marasmane was proposed, and likely marasmic acid to the Clavicorane-like sesquiterpenoid was achieved previously.[22] Therefore, these PTS reactions may have great potential in the combinatorial biosynthesis of natural products for bioactive lead discoveries as in the field of polyketides.[23]

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**Supporting Information Available:** $^1$H NMR spectra of **1**, **2a**, **2b**, **2c**, **2d**. **3**, **4**, **5**, **6**, and **7**, and X-ray crystallographic data in CIF format of **1** and **2d**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) No inhibitory activities were observed for compounds **1**−**3** against *Escherichia coli* CMCC44103, *Bacillus subtilis* CMCC63501, *Staphylococcus aureus* CMCC26003, and *Candida albicans* AS2.538 at 50 $\mu$g/mL by using ampicillin sodium and amphotericin B as positive controls and against HeLa cells (growth inhibitory rates <10%) at 20 $\mu$g/mL in the MTT assays.

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