Unique metabolites of *Pestalotiopsis fici* suggest a biosynthetic hypothesis involving a Diels–Alder reaction and then mechanistic diversification†

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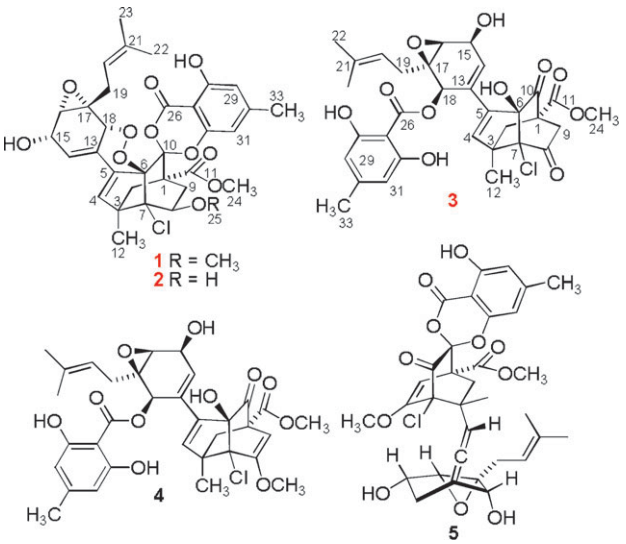
Chloropupukeanolides A (1) and B (2), unprecedented spiroketal peroxides, and chloropupukeanone A (3), three highly functionalized metabolites featuring a chlorinated pupukeanane core, were isolated from an endophytic fungus *Pestalotiopsis fici*, with 1 showing significant anti-HIV-1 and cytotoxic effects.

Pupukeanane sesquiterpenoids possessing the unique tricyclo[4.3.1.0^{3,7}]decane skeleton have mainly been isolated from marine sponges as isocyanates, thiocyanates and isothiocyanates.[1–7] The only examples from terrestrial sources are nemorosonol, a polyisoprenylated pupukeanane from the fruits of *Clusia nemorosa* and the leaves of *Garcinia bracteata*,[8,9] and chloropupukeananin (**4**), the first chlorinated derivative from the plant endophytic fungus *Pestalotiopsis fici*.[10]

The plant endophytic *Pestalotiopsis* spp. are well-known as a source of bioactive natural products.[11–19] Our prior work on *P. fici* (AS 3.9318 = W106-1) grown in different fermentation cultures led to the identification of bioactive metabolites with diverse structures,[10,20,21] such as chloropupukeananin (**4**) and its biosynthetic Diels–Alder precursors (**6** and **7** in Scheme 1).[10] To identify the key Diels–Alder intermediates and minor active products, the fungus was re-fermented on a larger scale on rice, in which **4** was initially isolated. The crude extract showed inhibitory effects on HIV-1 replication in C8166 cells and cytotoxicity against the human tumor cell lines, HeLa, MCF-7 and MDA-MB-231. Bioassay-guided fractionation of the extract afforded chloropestolide A (**5**),[22] a spiroketal with a novel framework as the antitumor principle. In addition, two spiroketal peroxides with an unprecedented skeleton named chloropupukeanolides A (**1**) and B (**2**), and a new analogue of **4**, chloropupukeanone A (**3**), were also isolated from the same extract with **1** as the anti-HIV-1 principle. Details of the structure elucidation, plausible biogenesis, and bioactivities of **1–3** are reported herein.



The molecular formula of chloropupukeanolide A (**1**) was determined to be $C_{33}H_{35}ClO_{11}$ by HRESIMS (*m*/*z* 665.1744 $[M + Na]^+$; Δ + 1.6 mmu). The NMR spectroscopic data of **1** revealed some structural features similar to those present in **4**, including the fragments of a tricyclo[4.3.1.0^{3,7}]decane, an isoprenylated 2,3-epoxycyclohex-5-en-1,4-diol (ECH), and a 2,6-dihydroxy-4-methylbenzoate (DMB) unit, which were confirmed by interpretation of the ^{1}H–^{1}H COSY and HMBC data. However, significant differences in chemical shifts were also observed for some signals corresponding to the tricyclo-[4.3.1.0^{3,7}]decane moiety in **1** compared to **4**. Specifically, the resonances for the C-8/C-9 olefin in **4** were replaced by those for a methine (δ_H/δ_C 4.15/69.2) and a methylene unit (δ_H/δ_C 2.08; 3.15/34.3) in the spectra of **1**, suggesting reduction of this olefin. Such observation was also confirmed by relevant ^{1}H–^{1}H COSY and HMBC correlations from H-8 to C-3, C-7 and C-9, and from H_2-9 to C-1, C-2, C-7, C-8, C-10 and C-11. HMBC cross-peaks from H-14 to C-5 and H-4 to C-13 led to the connection of the isoprenylated ECH to the tricyclo-[4.3.1.0^{3,7}]decane ring in **1** *via* the same C-5–C-13 linkage as in **4**. The exchangeable proton at δ_H 4.44 in **1** was assigned to be C-15–OH based on COSY correlation between this proton and H-15, whereas the other one at δ_H 10.2 was assigned as the phenolic C-28–OH by comparison of the chemical shifts for C-28 (δ_C 161.6) and C-32 (δ_C 157.9) in **1** with those in **4** (δ_C 162.0 for both carbons). The resonance for an oxygenated sp^3 quaternary carbon was observed at δ_C 102.1 in the

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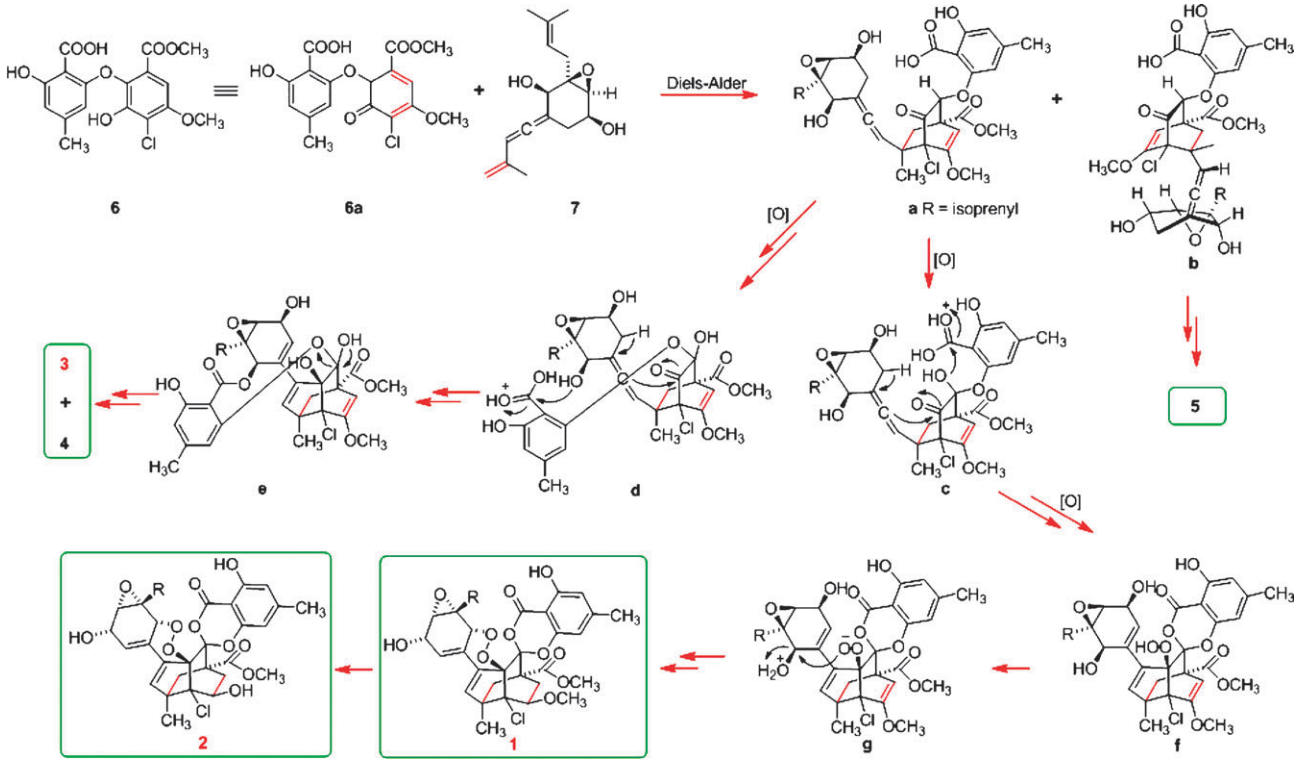

Scheme 1 Plausible biosynthetic pathways for compounds **1–5**.

spectrum of **1**, instead of a ketone carbon at δ_C 196.4 in **4**, suggesting that the C-10 ketone functionality was reduced to a ketal carbon, which was supported by the difference in chemical shift for C-10 in **1** and **5** (δ_C 97.9). The upfield chemical shifts for C-26 (δ_C 162.4 in **1** *vs.* 170.9 in **4**) and C-32 (δ_C 159.9 in **1** *vs.* 162.0 in **4**) indicated that they were each connected to one of the C-10 oxygen atoms *via* an ether and an ester linkages, completing the 1,3-dioxan-4-one ring that spirally joined the tricyclo-[4.3.1.$0^{3,7}$]decane unit at C-10, like that which appeared in **5**.[22] The downfield shifts for the oxygenated carbons C-6 (δ_C 108.4 in **1** *vs.* 86.5 in **4**) and C-18 (δ_C 79.4 in **1** *vs.* 70.9 in **4**), and the unsaturation requirement for **1** required the presence of a peroxide linkage between the two carbons. This assignment was partially supported by the downfield shifts for C-7, C-10 and C-13, which were close to the peroxide in space. Collectively, these data permitted completion of the gross structure of **1**.

The relative configuration of **1** was assigned on the basis of NOESY correlations and by comparison with those of **4** and **5**.[10,22] The NOESY data of **1** (Fig. 1) suggested that the isoprenylated ECH, tricyclo[4.3.1.$0^{3,7}$]decane, and the 1,3-dioxan-4-one substructures in **1** possess the same relative configurations as their counterparts in **4** and **5**, except for the stereogenic center C-8. NOESY correlations of H-8 with H-9a and H_3-12, and H-2a with H_3-12, indicated that these protons are all pseudoaxially oriented with respect to the corresponding six-membered ring, whereas those from H-31 to H-9b and H_3-25 revealed their proximity in space, establishing the relative configuration of C-8 as shown. The absolute configuration of **1** was proposed as 1*S*, 3*R*, 6*S*, 7*R*, 8*R*, 10*R*, 15*S*, 16*S*, 17*S* and 18*R* on the basis of above results, and by analogy to those of **4** and **5**, which were secured by X-ray crystallography.[10,22]

Chloropupukeanolide B (**2**) was assigned the molecular formula $C_{32}H_{33}ClO_{11}$ by HRESIMS (*m*/*z* 651.1626 $[M + Na]^+$; δ −2.2 mmu), which is 14 mass units less than that of **1**. The NMR data of **2** revealed nearly identical structural features to those of **1**, except that the C-25 methyl group (δ_H/δ_C 3.45/49.4) was replaced by a proton at δ_H 5.85 (OH-8), consistent with its HRESIMS data. Analysis of the NOESY data of **2** revealed the same relative configuration as **1**, implying that its absolute configuration could be deduced as shown by analogy to **1**.

The elemental composition of chloropupukeanone A (**3**) was established as $C_{32}H_{33}ClO_{11}$ by HRESIMS (*m*/*z* 651.1592 $[M + Na]^+$; Δ + 1.2 mmu). The ^1H and ^{13}C NMR spectra of **3** showed resonances for substructures similar to those present

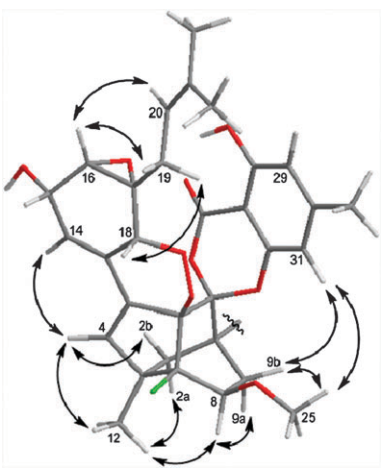

Fig. 1 Key NOESY correlations for chloropupukeanolide A (**1**).

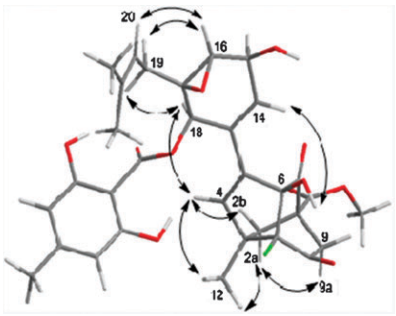

Fig. 2 Key NOESY correlations for chloropupukeanone A (**3**).

Table 1 Cytotoxicities (IC_{50}, μM) of compounds **1–3**

Compound	HeLa	MCF-7	MDA-MB-231
1	16.9	15.5	15.9
2	63.7	50.9	>100
3	38.2	31.8	73.1

in **4**, except those for the tricyclo[4.3.1.$0^{3,7}$]decane moiety. The resonances for the C-8/C-9 olefin and C-25 methoxy group attached to C-8 in **4** were replaced by those for a ketone carbon (δ_C 198.6) and a methylene unit (δ_H/δ_C 2.86; 3.15/47.0) in the spectra of **3**, and these observations were supported by HMBC correlations from H_2-9 to C-1, C-2, C-7, C-8, C-10 and C-11. Compound **3** was assigned the same relative configuration as **4** on the basis of NOESY data (Fig. 2) and by comparison to that of **4**, whereas the absolute configuration of **3** was presumably the same as that of **4**.

Compounds **1–3** were tested for *in vitro* anti-HIV-1 activity. **1** showed an inhibitory effect on HIV-1 replication in C8166 cells, with an EC_{50} value of 6.9 μM (the positive control, indinavir sulfate, showed an EC_{50} value of 8.81 nM). **1–3** were also evaluated for cytotoxicity against the human cancer cell lines, HeLa, MCF-7 and MDA-MB-231 (Table 1). Compound **1** showed significant cytotoxicity against the three cell lines, with IC_{50} values of 16.9, 15.5 and 15.9 μM, respectively.

Compounds **1** and **2** are chlorinated pupukeananes featuring an unprecedented spiroketal peroxide skeleton. Structurally, **1** and **2** incorporated the unique tricyclo[4.3.1.$0^{3,7}$]decane core, which not only spirally joined the DMB-originated 1,3-dioxan-4-one moiety at C-10, but also formed a six-membered peroxide with the ECH unit, completing a highly complex octacyclic structure, whereas compound **3** is a new analogue of **4**. Biogenetically, **6** and **7** discovered in both previous work and the current study, could be the Diels–Alder precursors, not only for **4** and **5**,[10,22,23] but also for **1–3** (Scheme 1). The discovery of **1–3** from *P. fici* may provide evidence for the biosynthetic pathways initially proposed for **4** and **5**.[10,22]

Compound **1** is not only structurally unique with an unprecedented skeleton, but also showed a significant anti-HIV-1 effect, and cytotoxicity against the HeLa, MCF-7 and MDA-MB-231 human tumor cell lines. These results strongly argue that further efforts are necessary to maximize the metabolic potential of this fungus to identify other "missing" Diels–Alder building blocks and active end products.

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