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ISOLATION AND IDENTIFICATION OF TWO NEW BIOLOGICALLY ACTIVE NORDITERPENE DILACTONES FROM *ASPERGILLUS WENTII*

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Key Word Index—*Aspergillus wentii*; mycotoxin; norditerpene dilactone; plant growth inhibition; X-ray crystallography.

Abstract—Two new biologically-active norditerpenoid dilactones were purified from culture extracts of *Aspergillus wentii* and assigned the trivial names wentilactone A and wentilactone B. The absolute chemical structure of wentilactone A was determined by single crystal X-ray diffraction and circular dichroism. The structure of wentilactone B was determined by ^{1}H and ^{13}C NMR analyses. Wentilactone A had an LD_{50} of 7.0 mg/kg when administered orally to 1-day-old chickens. Both metabolites inhibited growth in wheat coleoptile bioassays.

INTRODUCTION

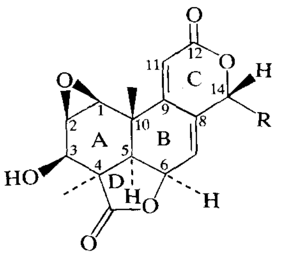
In the last decade several nor- and bisnorditerpenoid dilactones have been isolated from various *Podocarpus* species, and most have been shown to exhibit plant-growth regulating properties. Galbraith *et al.* [1] studied the relative activities of the podolactones and related compounds in the pea-stem bioassay, and they found podolactone E (**1**) to be the most inhibitory, followed by inumakilactone B (**2**). Singh *et al.* [2] studied the effects of six of these naturally-occurring lactones on housefly larvae, and they also found podolactone E to be the most active. In 1976 Ito and Kodama [3] published an extensive review of the literature on these compounds.

Using the screening program developed by Kirksey and Cole [4], we isolated a toxigenic fungus identified as *Aspergillus wentii* Wehmer (NRRL 6435) from peanuts which had molded during storage. We report here the isolation and identification of two new biologically active metabolites of the podolactone group purified from extracts of *A. wentii* cultures. These metabolites are given the trivial names wentilactone A (**3**) and wentilactone B (**4**), and their plant-growth inhibiting porperties are compared with those of podolactone E and inumakilactone B.

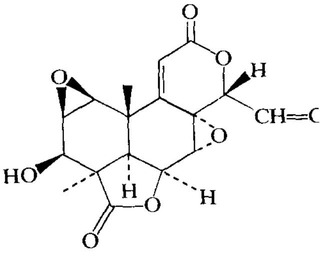
RESULTS AND DISCUSSION

Both **3** (mp 258°) and **4** (mp 260°) readily crystallized as cubes from a solution of MeOH–EtOAc (1 : 1). Crystals of each compound were soluble in H_2O and Me_2CO, sparingly soluble in MeOH, and insoluble in hexane, C_6H_6, EtO, $CHCl_3$, and EtOAc. TLC of **3** revealed a brown spot at R_f 0.18 that quenched under longwave UV light. **4** appeared as a yellow fluorescent spot under long-wave UV light at R_f 0.12.

UV data (Table 1) indicated the presence of a diene lactone system such as that found in antibiotic LL-Z1271α (**5**) [5, 6]. The IR spectrum of **3** showed major absorptions at 3500, 1780, and 1710 cm^{-1}, which are indicative of hydroxy, γ-lactone, and δ-lactone moieties, respectively. The HRP mass spectrum of **3** showed a molecular ion peak at *m/e*



1 Podolactone E R = CH═CH$_2$
3 Wentilactone A R = H

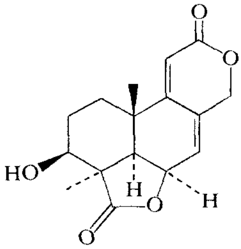


2 Inumakilactone B

Table 1. CD and UV spectral data of wentilactones A and B

	CD	UV
Wentilactone A	$[\theta]_{259}^{\mathrm{MeOH}} = -8.23\times10^{3}\,\frac{\mathrm{deg\ cm}^{2}}{\mathrm{dmol}}$	$\varepsilon_{259}^{\mathrm{MeOH}} = 1.34\times10^{4}\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$
Wentilactone B	$[\theta]_{260}^{\mathrm{MeOH}} = -8.32\times10^{3}\,\frac{\mathrm{deg\ cm}^{2}}{\mathrm{dmol}}$	$\varepsilon_{259}^{\mathrm{MeOH}} = 1.40\times10^{4}\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$

304.0914 (M^+), which was consistent with the calculated formula $C_{16}H_{16}O_6$ (calc. mass of 304.0946). **4** had an M^+ at *m/e* 290.1166 and the formula $C_{16}H_{18}O_5$ (calc. mass of 290.1153).



4 Wentilactone B

Preliminary X-ray diffraction experiments indicated that the symmetry of the clear crystals of **3** was $P2_12_12_1$, since reflections with h 0 0, $h = 2n+1$; 0 k 0, $k = 2n+1$; and 0 0 1, $1 = 2n+1$ were not observed. The cell constants found were $a = 8.908$ (1), $b = 10.391$ (2), and $c = 14.703$ (3) Å with $Z = 4$ for a calculated density of 1.48 g/cm^3. A labelled perspective drawing of the x-ray model is given in Fig. 1 [7]. Tables 2–4 which list fractional coordinates and temperature parameters, bond distances, and bond angles, respectively, are available as a supplementary publication [8].

Since **3** has a rigid, compact structure, the conformations of the various rings deviate somewhat from ideal models. As can be seen in Fig. 1, rings A, B, and D form a concave surface, with O-15, O-16, C-19, C-20, and O-22 having β orientations on the inside of the surface, while C-18 has an α orientation. Ring A exists in a distorted boat conformation very similar to an identical ring in inumakilactone A [9]. A distorted sofa conformation is the best description for ring B, with C-10 being 0.68 Å out of the best plane formed by C-5, C-6, C-7, C-8, and C-9. Ring C is also in a distorted sofa conformation, with C-14 being 0.47 Å out of the best plane formed by C-8, C-9, C-11, C-12, and O-13. Since C-5 is 0.53 Å out of the best plane formed by C-4, C-6, C-19, and O-22, the γ-lactone is in an envelope conformation. The only hydrogen bond in the crystal structure is intramolecular involving O-16, H-16, and O-21, with an O-16–O-21 distance of 2.78 Å.

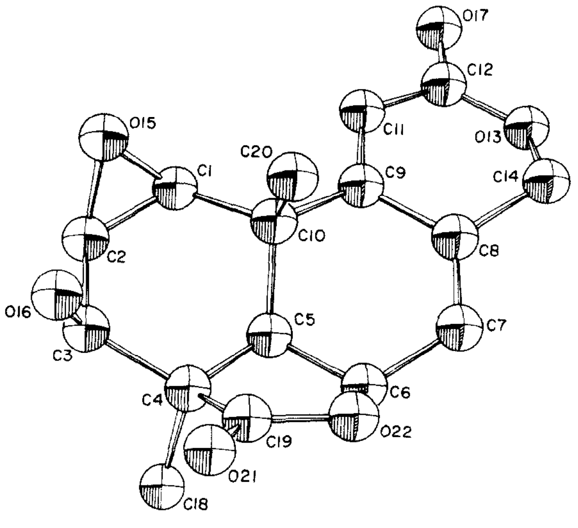
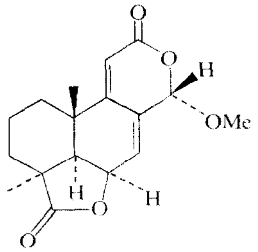


Fig. 1. A perspective drawing of wentilactone A with the correct absolute stereochemistry. Hydrogens have been omitted for clarity.



5 LL-Z1271α

The absolute configurations of **3** and **4** are as shown because of the large negative Cotton effect at 259 nm (Table 1) [10, 11].

The assignment of the ^1H NMR spectrum of **3** (Table 5) was relatively straightforward based on the chemical shifts, coupling constants, and spin-decoupling experiments. The ^{13}C chemical shifts of **3**

Table 5. ^1H NMR Chemical shifts and coupling constants of wentilactones A and B

Proton	Wentilactone A*†	Wentilactone B*†
1	3.63 (4.4)	1.3–2.3
2	3.38 (4.4, 6.1)	1.3–2.3
3	4.30 (6.1)	4.98
5	2.22 (5.6)	2.07 (5.4)
6	5.09 (5.6, 4.4)	5.19 (5.4, 4.4)
7	6.36 (4.4, 1.5)	6.35 (4.4, 1.7)
11	6.22 (1.5)	5.74 (1.7)
14	5.04	4.98
18	1.10	1.11
20	1.38	1.30

* Chemical shifts in ppm downfield from TMS.

† Coupling constants in parentheses, in Hz.

are given in Table 6. ^{13}C chemical shifts were assigned on the basis of the sford spectrum, trends in chemical shifts reported previously [12], and model compounds [13].

The ^{13}C spectrum of **4** (Table 6) contained 16 peaks in addition to those of the solvent, indicating the same number of carbon atoms in **3** and **4**. However, there were significant differences in the two spectra. First, the carbonyl assigned to the five-membered lactone in **3** was shifted downfield in **4** by ~5.0 ppm. The remainder of the peaks in the downfield region of the spectrum of **4** were similar to those in **3**. Second, the two carbons assigned to the epoxide in **3** were not present in **4**. Instead, two additional peaks appeared in the aliphatic region of the spectrum of **4**. Furthermore, sford spectra indicated that these two carbons are methylene carbons. Finally, the two methyl carbons in **4** were shifted downfield from their corresponding positions in **3**.

The ^{1}H spectrum of **4** (Table 5) likewise differed from that of **3** in several respects. The two protons assigned to the epoxide in **3** were absent in **4**, and a four-proton multiplet in the region 1.3–2.3 ppm was present in **4**. In addition, the signal assigned to H-3 in **3** was shifted downfield in **4**, whereas the signal assigned to H-11 in **3** was shifted upfield by ~0.5 ppm in the spectrum of **4**. The remainder of the ^{1}H spectrum of **4** was similar to that of **3**.

All the above data indicated that the structure of **4** was very similar to that of **3**. However, it was clear that **4** did not contain the epoxide ring. Thus, the data suggest that **4** has the structure as shown. The differences in the chemical shifts between **3** and **4** can be easily accounted for, since in **3** the six-membered ring containing the epoxide is locked into a boat conformation, whereas in **4** the ring is in a chair conformation.

Wentilactone A had an LD_{50} of 7 mg/kg in 1-day-old chickens. Shortly after dosing, the chicks exhibited severe equilibrium problems characterized by a tendency to fall forward. At the highest dosage level all animals were dead within 2 hr of dosing, whereas at the lowest level, all chicks survived and appeared to be fully recovered in 3 days. Wentilactone B was not toxic to 1-day-old chickens when administered orally up to 250 mg/kg. Limited supply of material precluded dosing of **4** at higher levels.

Table 6. ^{13}C NMR chemical shifts for wentilactones A and B

Carbon	Wentilactone A*	Wentilactone B*
1	50.20	39.63
2	54.69	36.33
3	66.78	63.22
4	48.21	42.24
5	47.72	45.53
6	69.49	71.30
7	121.82	121.64
8	131.15	131.38
9	156.45	158.17
10	35.57	35.56
11	111.90	111.85
12	162.95	163.16
14	68.81	69.32
18	18.65	23.15
19	175.84	180.70
20	24.85	27.37

* Chemical shifts in ppm downfield from TMS.

Podolactone E (**1**) and inumakilactone B (**2**), kindly provided by Dr. M. N. Galbraith, were used to compare their plant-growth-regulating activities with wentilactones A (**3**) and B (**4**). The results (Table 7), particularly at 10^{-5} M, showed that **3** was comparable in activity to **1** and **2**, whereas **4** was inactive. We, therefore, conclude from both the animal and plant studies that the C-1, C-2 epoxide is important for biological activity, since in each test wentilactone B is much less active than compounds containing that epoxide. This supports the conclusions reached by Galbraith *et al.* [1] in structure–activity studies of several members of the podolactone group in which they showed greater activity to be associated with compounds containing a C-1, C-2 epoxide or double bond. Hayashi and Sakan [14] also conducted structure–activity studies of various nor- and bisnorditerpenoid dilactones. It is interesting that the compounds showing greatest activity did not contain an epoxide between C-1 and C-2, but rather the smallest number of polar substituents in the molecule. Unfortunately, because of nonavailability of material, we could not compare the activities of **3** and **4** with the compounds found most active in their study.

Brown and Sanchez [15] reviewed evidence suggesting a dual ecological role for compounds of the podolactone group: (1) act as toxins which enable *Podocarpus* spp. to resist insect predator attack; (2) represent allelopathic substances which control patterns of plant colonization around *Podocarpus.* They further suggested that these compounds may be biosynthesized for the *Podocarpus* plants by mycorrhizal fungi. The rationale for such an argument is linked to a report [5] of an antifungal compound LL-Z1271α (**5**) belonging to the podolactone family and isolated from a fungus identified only as *Acrostalagmus* sp. (= *Verticillium*). Fungi assigned to the genus *Verticillium* are usually isolated from soil, often inhabiting the rhizosphere or infecting plant roots. Likewise, *A. wentii* is frequently isolated from soil and is one of the mold species commonly associated with the roots of pineapple plants in Hawaii and with the subterranean stems of *Coptis japonicus*, a drug plant [16]. It is interesting that the fungal metabolites, **3–5**, have no carbon side-chain at C-14, in contrast to the other members of the podolactone family, which were all isolated from *Podocarpus*. Therefore, it seems possible that the podolactones may be plant-altered fungal metabolites.

Table 7. Growth inhibiting activity of wentilactones A, B, podolactone E and inumakilactone B in wheat coleoptile bioassays (*Triticum aestivum* L. cv Wakeland) expressed as per cent inhibition compared to controls

	10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M
Wentilactone A	81	81	48	0
Wentilactone B	100	81	0	0
Podolactone E	82	64	55	0
Inumakilactone B	96	82	62	0

EXPERIMENTAL

Isolation of toxigenic fungus. The toxigenic strain of *A. wentii* (NRRL 6435) was isolated from molded warehouse peanuts using potato dextrose agar (PDA) plates. Peanuts were surface-sterilized under vacuum for 1 min with 2% NaClO and incubated for 4 days at 25°. The fungus was maintained on PDA plates at 5°, and toxigenicity was determined using the method of Kirksey and Cole [4].

Production and purification of metabolites. The mold was cultured for 21 days at 27° in 40 Fernbach flasks (2.8), each containing 100 g shredded-wheat supplemented with 200 ml Difco mycological broth (adjusted to pH 4.8) plus YES medium [17]. To extract the metabolites, 400 ml $CHCl_3$ was added to each culture flask. The flasks were heated on a steam bath for 10 min, and the contents were homogenized in a Waring Blendor. The extract was filtered through cheesecloth into a 4-l. separatory funnel. The $CHCl_3$ phase was recovered, vacuum-filtered through dry Na_2SO_4, and concd. The material in the cheesecloth was re-extracted with an equal vol. of $CHCl_3$; and the extract was filtered through Na_2SO_4, concd, and combined with the first extract.

The crude extract was fractionated on a 9.5 cm i.d. × 17 cm silica gel 60 column packed as a slurry in toluene. The extract was eluted sequentially with 3 l. toluene, 3 l. Et_2O, and 2 l. each EtOAc, Me_2CO, and MeOH. Since both the EtOAc and Me_2CO fractions were toxic to 1-day old chickens, they were combined, concd, redissolved in C_6H_6, and applied to a 4.5 cm i.d. × 40 cm silica gel column packed in benzene. The column was eluted with 2 l. C_6H_6 and placed on an automatic fraction collector. The column was further eluted with a linear gradient from C_6H_6 to EtOAc, followed by a gradient from EtOAc to Me_2CO. Of the 460 fractions (17 ml each) collected, fractions 247–291 were toxic. These were combined, evapd to dryness, redissolved in EtOAc, and applied to a 3.5 cm i.d. × 40 cm Si gel column packed in EtOAc. A gradient from EtOAc to 25% Me_2CO in EtOAc followed elution with 1 l. EtOAc. Fractions 16–46 were combined, and as evapn of the solvent neared completion, crystals formed in the flask. The crystals were collected and found to be toxic in the chick bioassay. TLC of these toxic crystals revealed the presence of two major metabolites. The crystals were dissolved in a minimal amount of Me_2CO and applied to a Florisil column (2 cm i.d. × 50 cm) packed in C_6H_6. A gradient from C_6H_6 to EtOAc separated the two metabolites; fractions 54–84 contained a relatively non-toxic compound (wentilactone B); fractions 134–190 contained the toxin (wentilactone A); and fractions 85–133 contained a mixture of the two.

Animal and plant bioassay. Purification of the metabolites was monitored using 1-day-old chickens dosed orally via crop intubation. Crude samples were administered in corn oil suspension at 1 ml per chicken [4]. All purified crystalline material was administered in H_2O solution at 1 ml per chicken. For determination of the LD$_{50}$ of wentilactone A, the toxin was administered in H_2O solution to four chickens each at concns of 100, 200, 400, and 800 μg per chicken. Weil's tables were used to calculate the LD$_{50}$ [18]. Wentilactone B was dosed at levels up to 250 mg/kg (10 mg/chicken).

Wheat coleoptile sections were cut 4 mm long from etiolated seedlings (*Triticum aestivum* L. cv Wakeland) grown on moist sand for 4 days at 22 ± 1° in the dark [19]. The seedlings were placed in a Van der Weij guillotine, and the apical 2-mm section was cut and discarded; the next 4-mm section was cut and retained for bioassay. Solutions of the purified metabolites, podolactone E, and inumakilactone B were made up in phosphate–citrate buffer (pH 5.6) plus 2% sucrose [20] at concns of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M. The initial stock solution of each compound was made at 10^{-3} M by dissolving each in 50 μl Me_2CO [21] and adjusting the vol. to 10 ml with buffer solution. Each assay tube contained 2 ml of test solution plus 10 coleoptile sections. All manipulations with the coleoptiles were carried out under a green safelight. Coleoptiles were incubated and rotated at 0.25 rpm in a roller tube apparatus, in the dark, for 24 hr at 22°. Following incubation, the sections were measured by projecting their images (× 3) from a photographic enlarger [22]. All data were statistically analysed [23].

Physical and chemical analyses. TLC plates coated with 0.5 mm of Si gel G-HR were used to analyse the metabolites. The developing solvent system was $CHCl_3$–Me_2CO–MeOH (17 : 2 : 1), and visualization was achieved by spraying plates first with *p*-dimethylaminobenzaldehyde in EtOH (1 g/100 ml), then with 50% ethanolic H_2SO_4 followed by heating for 10 min at 125°. The plates were then viewed under long-wave UV light.

Mps are uncorr.; UV spectra in MeOH; IR spectra as a thin film coated on to KBr windows. HRP mass spectral analyses were made with an AEI MS-902 double focus mass spectrometer. Samples were introduced into the ion source by the direct probe method, and ionized by electron impact at 70 eV at an ion-source temp. of 200°.

Single crystal X-ray data were collected at room temp. with a Syntex P$_{2_1}$ diffractometer using graphite monochromated CuKα ($\lambda = 1.5418$ Å) radiation and $2\theta \leq 114°$. Each unique reflection was measured using a variable-speed ω-scan technique. Of the 1083 unique reflections measured, 1038 (96%) were considered observed ($I > 3\sigma_I$). Data were corrected for Lorentz, polarization, and background effects but not absorption. Positions for 19 of the 22 non-hydrogen atoms were found using a multi-solution tangent formula approach [24]. Positions for the remaining nonhydrogen atoms and hydrogen atoms were found using Fourier analyses and least-squares refinements by minimizing $\sum\omega(|F_o| - |F_c|)**2$ with $\omega = (1/\sigma F_o)**2$ [25]. The final unweighted residual after full-matrix least-squares refinements employing anisotropic temp. parameters for the nonhydrogen atoms and fixed isotropic temp. parameters for the hydrogens was 0.035.

Proton and natural abundance ^{13}C NMR spectra were obtained on a Varian Associates XL-100-12 spectrometer equipped with the 620-L disk data system and Gyro Code accessory. Wentilactone A was run as a saturated solution in DMSO-d_6 (5 mm tube) and wentilactone B (9 mg/400 μl) in a 1 : 1 mixture of $CDCl_3$ and DMSO-d_6. The deuterium signal from the solvent was used as the internal lock, and TMS was used as the internal reference. Proton spectra were run in the CW mode, and homonuclear-spin-decoupling experiments were used to aid in the assignments. The ^{13}C NMR spectra were run in the FT mode with the following parameters: 2 kHz proton noise decoupling; spectral width 5 kHz; pulse width 30; pulse repetition 2 sec; data points 8 K; and exponential weighting function − 1.0. Sford ^{13}C spectra were obtained to aid in the assignment of the ^{13}C spectra.

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