Dichlorinated Pulvinic Acid Derivative from a Malaysian *Scleroderma* sp.

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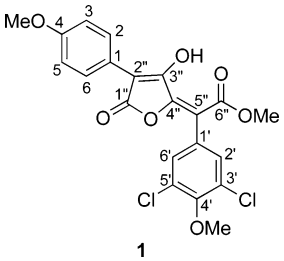
A new dichlorinated pulvinic acid derivative, methyl-3′,5′-dichloro-4,4′-di-*O*-methylatromentate (**1**), was isolated from the fruiting body of a *Scleroderma* sp. The structure of **1** was determined using spectroscopic methods, and an X-ray analysis was carried out for confirmation of the structure. Compound **1** was found to display moderate antimicrobial activity against *Bacillus subtilis*.

Scleroderma spp., otherwise known as "earthballs" or "poison puff balls", occur as ecto-mycorrhizal associates of a wide range of trees, including oak and eucalyptus,[1] or as saprotrophs in soil or rotting wood.[2] The fruiting bodies are formed at the surface of the soil, or just below, and are reminiscent of a potato in appearance.[1,3] They have a brown outer peridium and when the spores are mature a dark, blackish purple interior. About 25 species of *Scleroderma* have been described worldwide,[2] and at least four species are known to be poisonous: *S. albidum, S. areolatum, S. cepa*, and *S. citrinum*. Symptoms of poisoning can occur within an hour of eating and include loss of consciousness, nausea, severe abdominal pains, vomiting, perspiration, generalized tingling sensations, spasms, cramps, paralysis, and anaphylactic shock.[1,3] The chemistry of the "earthballs" has not, however, been well studied.[3] In our search for biologically active natural products from Malaysian fungi, we discovered that the methanol extract of a *Scleroderma* sp. showed antibacterial activity against *Bacillus subtilis* (11 mm inhibition zone). Purification of this extract resulted in the isolation of a new pulvinic acid derivative, methyl-3′,5′-dichloro-4,4′-di-*O*-methylatromentate (**1**), as well as three known pulvinic acid derivatives.Compound



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1 was isolated as a minor compound by semipreparative HPLC and was obtained as an intense yellow colored amorphous solid. EIMS on 1 revealed the molecular ion as a 9:6:1 triplet at *m/z* 450/452/454. This was followed by HREIMS on the ion at *m/z* 450, which established a molecular formula of $C_{21}H_{16}O_7{}^{35}Cl_2$, indicative of 13 degrees of unsaturation. The 1H NMR spectrum (Table 1) showed only seven signals, three of these being methoxyl (δ_H = 3.85, 4-OCH_3, 3.96, 4′-OCH_3, and 3.89, H7″). Also present

Table 1. 1D and 2D NMR Data for Compound **1** in $CDCl_3$

position	δ_C	δ ^{1}H, multiplicity (J_{HH} Hz)	CIGAR
1	121.2 (C)		
2	129.5 (CH)	8.12 d (8.8)	4, 6, 3, 2″
3	114.0 (CH)	6.97 d (8.8)	4, 1, 5
4	159.7 (C)		
5	114.0 (CH)	6.97 d (8.8)	4, 1, 3
6	129.5 (CH)	8.12 d (8.8)	4, 2, 5, 2″
4-OCH_3	55.3 (CH_3)	3.85 s	4
1′	129.1 (C)		
2′	130.4 (CH)	7.19 s	4′, 6′, 3′, 5″
3′	129.4 (C)		
4′	152.4 (C)		
5′	129.4 (C)		
6′	130.4 (CH)	7.19 s	4′, 2′, 5′, 5″
4′-OCH_3	60.8 (CH_3)	3.96 s	4′
1″	165.6 (C)		
2″	105.8 (C)		
3″	158.0 (C)	13.42 (OH)	3″, 4″, 2″
4″	155.8 (C)		
5″	112.1 (C)		
6″	170.7 (C)		
CO_2CH_3	54.6 (CH_3)	3.89 s	6″, 5″

were three sets of signals (δ_H = 8.12−6.97) in the aromatic region, which integrated in total for seven protons, with four of the protons originating from a 1,4-disubstituted aromatic system (δ_H = 8.12, H2,6; J = 8.8 Hz, 6.97, H3,5; J = 8.8 Hz). The other aromatic signal was a 2H singlet (δ_H 7.19) suggestive of a symmetric tetrasubstituted aromatic ring. An additional NMR singlet at δ_H = 13.42 indicated a hydrogen-bonded hydroxyl proton. The ^{13}C NMR spectrum (Table 1) showed 17 signals. The three methoxyls at δ_C = 55.3 (C-4), 60.8 (C-4′), and 54.6 (C-7″) and probably two ester carbonyls at δ_C 165.6 and 170.7 were immediately apparent.

The carbon skeleton of **1** was in part deduced from analysis of the CIGAR and ^{13}C NMR experiments. Analysis of the CIGAR spectrum confirmed the presence of two aromatic rings, one of them 1,2,4,6-tetrasubstituted and the other 1,4-disubstituted, as well as a methyl ester functionality. The remaining signals were quaternary carbons, and only two CIGAR correlations were observed to that part of the molecule that lay between the aromatic rings. Consultation with the AntiMarin database[4] yielded structures for related compounds.[5,6] The NMR data were compared with those reported in the literature, and this suggested that the system linking the two aromatic rings was a hydroxylated furanone and that the compound was a pulvinic acid derivative.

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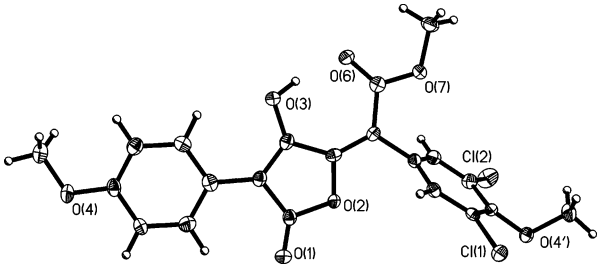

Figure 1. ORTEP diagram of methyl-3′,5′-dichloro-4,4′-di-*O*-methylatromentate (**1**).

With a working structure in hand, the NMR signals (Table 1) of compound **1** were assigned. Crystallization of **1** by slow evaporation of MeOH yielded needles of an intense yellow color suitable for X-ray analysis, which confirmed the structure of **1** (Figure 1). The new natural product was given the trivial name of methyl-3′,5′-dichloro-4,4′-di-*O*-methylatromentate. This is the first X-ray structure of an atromentate structure. The only other X-ray structure in this series is that of vulpinic acid.[7] Further confirmation of the structure came from the EIMS. The major fragment peak in the EIMS at 418/420/422 was indicative of formation of a dilactone peracetate derivative through loss of MeOH from the parent compound. This phenomenon had previously been seen in other vulpinic acid derivatives.[8]

In addition to the new dichlorinated pulvinic derivative, methyl-3′,5′-dichloro-4,4′-di-*O*-methylatromentate (**1**), three known compounds, 4,4′-dimethoxyvulpinic acid,[5] methyl-3′-chloro-4,4′-di-*O*-methylatromentate,[6] and methyl 4,4′-dimethoxyvulpinate,[5,9] were also isolated. The structures of these known compounds were identified by spectroscopic (^{1}H NMR, ^{13}C NMR, 2D NMR) and MS data measurement and by comparison with published values.

Dichlorinated pulvinic acids as natural products have been previously reported. These include methyl-2′,5′-dichloro-4,4′-di-*O*-methylatromentate,[5] in which the two chlorines are *para*-substituted, or dichloro-tri-*O*-methylxerocomic acid methyl ester,[6] where both aromatic rings are monosubstituted *ortho* to the methoxyl. Many halogenated pulvinic acid derivatives have also been synthesized for in vivo evaluation as potential anti-inflammatory agents.[10] Compound **1**, however, has not been reported as a synthetic product.

Compound **1** was found to be inactive (IC_{50} value 20.6 μM) against the P388 murine leukemia cell line, but when tested as an inhibitor of bacterial and fungal pathogens, was found to show moderate inhibition of *B. subtilis* (an inhibition zone of 4 mm was observed at a concentration of 40 μg). The larger zone of inhibition that had been initially seen in the crude extract (11 mm) was attributed to the two known compounds, 4,4′-dimethoxyvulpinic acid and methyl-3′-chloro-4,4′-di-*O*-methylatromentate, which showed inhibition zones of 8 and 7 mm, respectively, under identical assay conditions. No inhibition of *B. subtilis* has previously been reported for any chlorinated pulvinic acid derivatives.

Experimental Section

General Experimental Procedures. Melting points were obtained on a Reichert Hotstage melting-point apparatus. NMR experiments were recorded in $CDCl_3$ on a Varian INOVA 500 spectrometer at 23 °C, operating at 500 MHz using the signals of the residual solvent protons and the solvent carbons as internal references (δ_H 7.25 and δ_C 77.01 ppm for $CDCl_3$). IR spectra were recorded on a Shimadzu FTIR-8201 PC spectrometer. UV spectra were recorded on a Varian Cary 50 Probe UV−visible spectrophotometer. Electron impact mass spectra were obtained on a Kratos MS80RFA spectrometer, operating with a 4 kV accelerating potential, 70 eV, and a source temperature of 250 °C. Preparative HPLC was performed on a Shimadzu LC-4A instrument equipped with a UV spectrophotometric detector SPD-2AS (wavelength λ = 210 nm) and was carried out on a Phenomenex Luna C18, 10 × 250 mm, 5 μm column run at 5 mL/min. Solvents used for extraction and isolation were distilled prior to use. Cytotoxicity against P388 cells and antimicrobial activities were measured using a standard protocol.[11] X-ray crystallographic data were recorded on a Siemens P4 four-circle diffractometer using graphite-monochromated Mo Kα (λ = 0.71073 Å) radiation at the temperature indicated in Table S1 (see Supporting Information).

Fungal Material. Specimens were collected in the forest in Kuala Lompat, Pahang, in Malaysia in June 2004. A voucher specimen of this *Scleroderma* sp. has been deposited in the collection at the School of Chemical Sciences and Food Technology, Universiti Kebangsaan Malaysia (UKM-F5031). Identification of the fungal material was carried out by one of the authors (A.L.J.C.). The sample was identified by the characteristic tuberous earth ball fruiting body, differing from many other known *Scleroderma* sp. by the yellow color and thick rooting stalk.

Extraction and Isolation. Fresh fungal material, consisting of three fruiting bodies (12.53 g), was dried and then extracted repeatedly with MeOH (100 mL). The extract was dried under vacuum to yield an intense orange solid crude extract (1.479 g). The EtOAc-soluble extract was found to show good activity against *B. subtilis* in the antimicrobial assay. The crude extract was divided into MeOH-soluble (473 mg) and -insoluble fractions (1.006 g). The MeOH-soluble extract was found to show good activity against *B. subtilis* in an antimicrobial assay. A portion (50 mg) of the MeOH-soluble fraction was purified by HPLC using a C18 semipreparative column (Luna 5μm, 250 × 10.00 mm, ACN/H_2O, 60:40−80:20, with 0.05% TFA over 20 min, 5 mL/min, detection at 210 nm) and provided compound **1** (2.1 mg), 4,4′-dimethoxyvulpinic acid (3.1 mg), methyl-3′-chloro-4,4′-di-*O*-methylatromentate (1.6 mg), and methyl 4,4′-dimethoxyvulpinate (1.2 mg).

Methyl-3′,5′-dichloro-4,4′-di-*O*-methylatromentate (1): yellow needles (MeOH); mp 170−171 °C; UV (MeOH) λ_{max} (log ϵ) 277 (3.84), 399 (3.60) nm; IR (KBr disk) ν_{max} 1733, 1710, 1600, 1255, 1225, 1188, 1072, 1311 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; EIMS *m/z* (%) 450/452/454 $[M]^+$ (7/5/1), 418/420/422 (100/67/13), 362/364/366 (35/20/5), 306/308/310 (28/18/4), 175 (19), 147 (57), 119 (41); HREIMS *m/z* 450.0266 $[M]^+$ (calc for $C_{21}H_{16}{}^{35}Cl_2O_7$ 450.0273). Crystallographic data for compound **1** has been deposited with the Cambridge Crystallographic Data Centre.[12]

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Note Added after ASAP Publication. "Pyranone" was changed to "furanone" in the last sentence on the first page.

Supporting Information Available: ^{1}H NMR spectrum and X-ray data tables containing structure refinement, atomic coordinates, bond lengths and angles, anisotropic displacement parameters, hydrogen coordinates, torsion angles, and hydrogen bonds for compound **1** are available free of charge from the Internet at http://pubs.acs.org.

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(12) Copies of the data can be obtained free of charge, on application to the director, CCDC, 12 Union Road, Cambridge CB2 IEZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@code.cam.ac.uk).

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