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Dhilirolides A−D, Meroterpenoids Produced in Culture by the Fruit-Infecting Fungus *Penicillium purpurogenum* Collected in Sri Lanka

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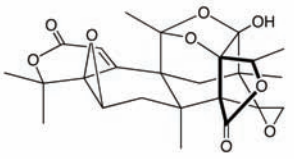
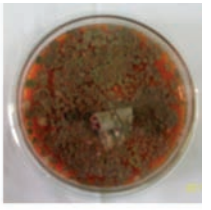
ABSTRACT

Averrhoa bilimbi



Dhilirolide A



Penicillium purpurogenum

Dhilirolides A (1) to D (4), a family of secondary metabolites with a putative meroterpenoid biogenetic origin and the unprecedented dhilirane and isodhilirane carbon skeletons, have been isolated from laboratory cultures of the fruit-infecting fungus *Penicillium purpurogenum* collected in Sri Lanka. The structures of 1 to 4 were elucidated by interpretation of NMR data and a single crystal X-ray diffraction analysis of 1.

The fruiting tree *Averrhoa bilimbi* is commonly found in home gardens in rural Sri Lanka, where it is referred to as 'biling' in Sinhalese. Mature *A. bilimbi* fruit is susceptible to infection by the fungus *Penicillium purpurogenum*. Infected areas initially appear as dark red spots on the bright green or yellowish surface of the fruit. Once established, the localized spot infections rapidly enlarge leading to the complete decay of the fruit.

A chemical investigation of laboratory cultures of *P. purpurogenum* has resulted in the isolation of four new secondary metabolites, dhilirolides A (**1**) to D (**4**),[1] that have a putative meroterpenoid biogenetic origin and the unprecedented dhilirane (**5**) and isodhilirane (**6**) carbon skeletons. Details of the isolation and structure elucidation of the dhilirolides are presented below.

An isolate of *P. purpurogenum* was obtained from infected *A. bilimbi* fruit collected in Nugegoda, Sri Lanka.[2] Laboratory cultures of *P. purpurogenum* were grown on the surface of potato dextrose agar in Petri dishes for 5 days at room temperature. A deep red pigment produced by the fungus was excreted into the agar during the culture period, mimicking the coloration of the fruit infection. The cultures were harvested by scraping the fungal mycelium off the agar surface before cutting the agar into small pieces that were extracted three times with fresh EtOAc. Evaporation of the combined EtOAc extracts in vacuo gave 1.4 g of a red solid that was subjected to a series of solvent/solvent partitioning steps (see Supporting Information for

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(1) Fungi are called 'dhilira' in Sinhalese, hence the name 'dhilirolides' to describe these Sri Lankan fungal-derived metabolites.

(2) The fungus was identified using morphological and reproductive characters (IMI no 357108) according to: Dahanayake, S.; Wijesundera, R. L. C. *J. National Science Council, Sri Lanka* **1994**, *22*, 23–24. A voucher specimen (ref no. 01-2010) is deposited at the Department of Plant Sciences.

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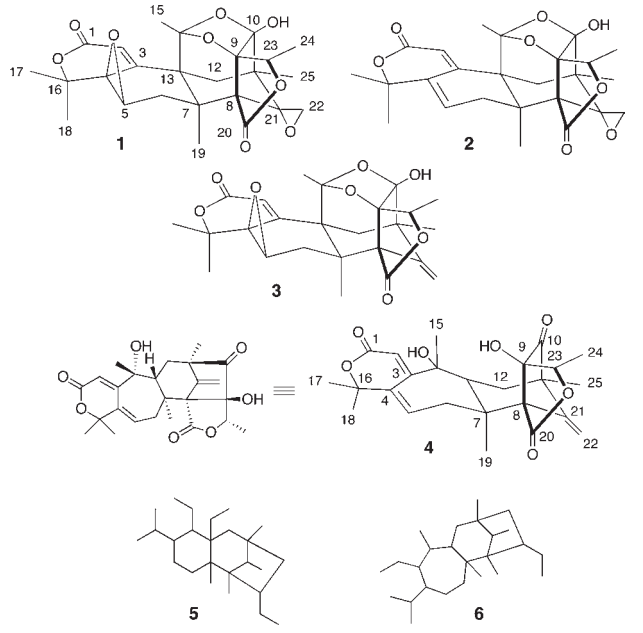
complete fractionation details). The CH_2Cl_2-soluble materials were fractionated by sequential application of Sephadex LH-20 chromatography, flash C_{18} reversed-phase chromatography, and C_{18} reversed-phase HPLC to give pure samples of dhilirolides A (**1**: 5.2 mg), B (**2**: 3.7 mg), C (**3**: 1.9 mg), and D (**4**: 2.0 mg).



Dhilirolide A (**1**) was obtained as optically active colorless crystals (mp 267−269 °C) that gave an $[M + H]^+$ ion in the HRESIMS at m/z 473.1796 appropriate for a molecular formula of $C_{25}H_{28}O_9$, requiring 12 sites of unsaturation. The ^{13}C NMR spectrum obtained for **1** contained 25 resolved resonances in agreement with the HRESIMS data (Table 1: Supporting Information). A detailed analysis of the $^1H/^{13}C$/gCOSY/gHSQC/gHMBC NMR data identified five methyl singlets [δ 0.74 (Me-25), 1.18 (Me-17), 1.25 (Me-19), 1.50 (Me-18), 1.51 (Me-15)], one methyl doublet [δ 1.42 J = 7.1 Hz (Me-24)], a trisubstituted olefin [δ 6.42 (H-2), 125.0 (C-2); 152.2 (C-3)], two ester/lactone carbonyls [δ 162.0 (C-1); 170.3 (C-20)], a trisubstituted epoxide [δ 55.6 (C-4); 3.68 (H-5), 55.9 (C-5)]; a 1,1 disubstituted epoxide [δ 64.0 (C-21); 2.36/2.90 (H-22β/H-22α), 45.6 (C-22)], two oxygenated tertiary carbons [δ 91.0 (C-9); 81.5 (C-16)], an oxygenated methine carbon [δ 4.73 (H-23), 80.9 (C-23)], two ketals [δ 105.7 (C-10); 107.7 (C-14)], four other quaternary carbons [δ 40.5 (C-7); 54.2 (C-8); 44.9 (C-11); 47.9 (C-13)], and two methylenes [δ 1.77/2.46 (H-6_{ax}/H-6_{eq}), 31.5 (C-6); 1.74/2.11 (H-12_{ax}/H-12_{eq}), 36.0 (C-12)]. The alkene, carbonyl, and epoxide functionalities described above accounted for 5 of the 12 sites of unsaturation indicated by the molecular formula, requiring that dhilirolide A had to contain seven additional rings.

Only one proton resonance (δ 7.39, s, OH-10) did not correlate to a carbon resonance in the HSQC spectrum of **1**. This OH-10 resonance correlated to the ketal carbon at δ 105.7 (C-10) in the HMBC experiment, consistent with the presence of a hemiketal. The two esters, two epoxides, and one OH accounted for seven of the nine oxygen atoms in the molecular formula. Since there were no further exchangeable protons or carbonyls, the remaining two oxygen atoms had to be present as ether linkages.

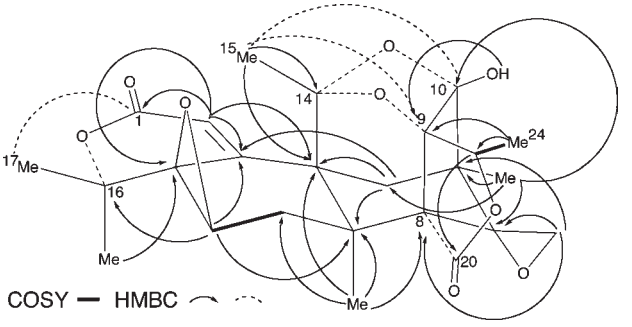


Figure 1. COSY and HMBC correlations used to assign the constitution of **1**.

Analysis of the COSY and HMBC data as illustrated in Figure 1 readily established most of the constitution of dhilirolide A (**1**). Of particular note, were three weak 4-bond HMBC correlations observed between Me-17 (δ 1.18) and C-1 (δ 162.0), and between Me-15 (δ 1.51) and both C-9 (δ 91.0) and C-10 (δ 105.7), that established the ester linkage between C-1 and C-16 and the ether linkages between the C-14 (δ 107.7) ketal carbon and both the C-9 oxygenated tertiary carbon and the C-10 hemiketal carbon. The HMBC and COSY data failed to identify the final substituents on either C-8 or C-20 and one site of unsaturation. Simply linking the unsatisfied valences on C-8 and C-20 generated a γ-lactone that provided the final ring required by the molecular formula, completing the constitution of **1**. The rigidity of **1**, resulting from its fused polycyclic structure, facilitated the assignment of the complete relative configuration via analysis of the observed ROESY correlations as shown in Figure 2.

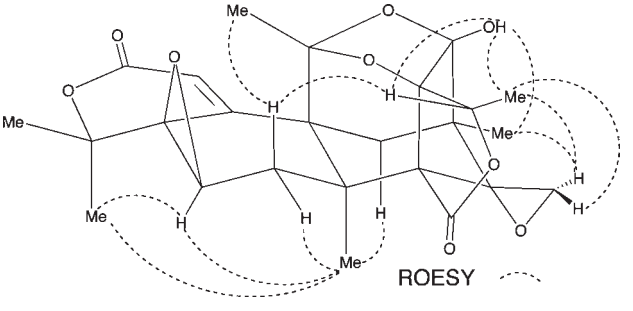


Figure 2. ROESY correlations used to assign the relative configuration of dhilirolide A (**1**).

The structure proposed for dhilirolide A (**1**) from analysis of its NMR data has a complex unprecedented carbon skeleton. In order to verify the proposed structure, dhilirolide A (**1**) was subjected to single crystal X-ray diffraction analysis. The material crystallizes with four crystallographically

independent molecules of dhilirolide A (**1**) in the asymmetric unit. The absolute configuration was established on the basis of the refined Flack *x*-parameter [$x = -0.06(11)$].[3] Additionally, the material crystallizes as a two component twin, with twin domains related by a 180° rotation about the [−1 1 0] reciprocal lattice axis. The ratio of major-to-minor twin components is 0.84:0.16. The ORTEP diagram shown in Figure 3 shows the proposed constitution and relative configuration of dhilirolide A (**1**) with its absolute configuration as 4*S*, 5*R*, 7*R*, 8*R*, 9*R*, 10*R*, 11*R*, 13*S*, 14*S*, 21*R*, 23*S*.

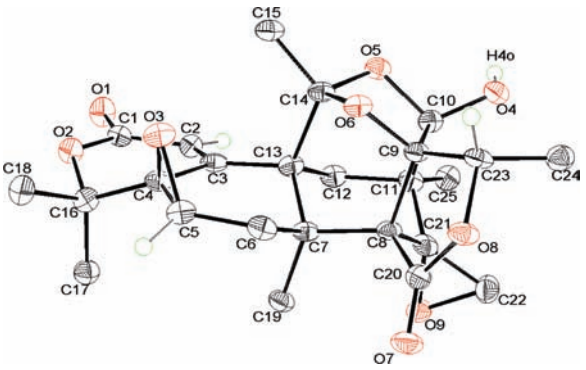


Figure 3. ORTEP diagram for dhilirolide A (**1**).

Dhilirolide B (**2**) was isolated as an optically active amorphous solid that gave an $[M + Na]^+$ ion at *m*/*z* 479.1725 in the HRESIMS appropriate for the molecular formula of $C_{25}H_{28}O_8$, that differs from that of **1** simply by the loss of an oxygen atom. Although the ^1H and ^{13}C NMR spectra of **2** were similar to those of **1**, the UV spectrum was markedly different. In dhilirolide A (**1**), a λ_{max} typical of a trisubstituted α,*β*-unsaturated lactone was observed at 235 nm, while, in **2**, the λ_{max} was shifted to 280 nm. Replacing the C-4/C-5 epoxide in **1** with a double bond in **2** would account for the loss of an oxygen and extend the conjugation from the enone in **1** to a dienone in **2**, satisfying the λ_{max} observed for **2**. Examination of the 1D and 2D NMR data (Table 1: Supporting Information) obtained for **2** revealed that the epoxide H-5 resonance (*δ* 3.68) in **1** had been replaced by an olefinic resonance at *δ* 6.15, that showed COSY correlations to the methylene resonances at *δ* 1.94 and 2.92 assigned to H-6_{eq}/H-6_{ax}. HMBC correlations between C-4 (*δ* 133.4) and H-2 (*δ* 5.83), H-6_{eq}/H-6_{ax} (*δ* 1.94/2.92), Me-17 (*δ* 1.50), and Me-18 (*δ* 1.54), and between H-5 (*δ* 6.15) and both C-3 (*δ* 150.0) and C-16 (*δ* 82.0), were consistent with the proposed structure.

Dhilirolide C (**3**) was isolated as an optically active amorphous solid that gave an $[M + Na]^+$ ion at *m*/*z* 479.1746 in the HRESIMS consistent with the molecular formula $C_{25}H_{28}O_8$, identical to the molecular formula of dhilirolide B (**2**). The UV spectrum observed for **3** (λ_{max} 239 nm) was similar to that of dhilirolide A (**1**) (λ_{max} 235 nm) indicating that it did not have the dienone moiety found in **2**. Comparison of the ^1H and ^{13}C NMR spectrum of dhilirolide C (**3**) with the corresponding spectra recorded for dhilirolide A (**1**) (Table 1: Supporting Information) revealed that the resonances assigned to the 1,1-disubstituted C-21/C-22 epoxide in **1** were absent. Instead, the ^1H NMR spectrum of **3** contained two new singlet resonances at *δ* 5.04 (H-22*β*) and *δ* 5.11 (H-22α), which both correlated to an olefinic carbon at *δ* 107.7 (C-22) in the HSQC spectrum and to a second olefinic carbon at *δ* 151.7 (C-21) in the HMBC spectrum. These observations showed that **1** and **3** differed simply by the replacement of the C-21/C-22 epoxide in **1** with a $\Delta^{21,22}$ exocyclic alkene in **3**. HMBC correlations observed between the H-22*β*/H-22α olefinic methylene resonances at *δ* 5.04 and 5.11 and the methine carbons at *δ* 61.3 and 50.4, assigned to C-8 and C-11, respectively, supported this assignment. Interestingly, although all the other structural features of **1** and **3** were identical, it was found that when the ^1H NMR spectrum of **3** was recorded in DMSO-d_6 many resonances were doubled or broadened and significantly shifted. This phenomenon was attributed to a slow conformational equilibrium. A single set of well-resolved resonances could be obtained when the NMR spectra for **3** were recorded in MeOH-d_4.

Dhilirolide D (**4**) was isolated as an optically active amorphous solid that gave an $[M - H]^-$ ion at *m*/*z* 441.1967 in the HRESIMS appropriate for a molecular formula of $C_{25}H_{30}O_7$. The molecular formula of **4** differed from the molecular formula of **1** by the addition of two hydrogen atoms and the loss of two oxygen atoms, and it required only 11 sites of unsaturation. Examination of the ^1H and ^{13}C NMR spectra recorded for dhilirolide D (**4**) revealed a close relationship to dhilirolides A−C (**1**−**3**), but also several significant structural and functional group differences. The UV (λ_{max} 276 nm) and $^1H/^{13}C$/COSY/HSQC/HMBC NMR data (Table 1: Supporting Information) obtained for **4** identified the C-1 to C-5 dienone substructure present in **2** and the $\Delta^{21,22}$ exocyclic alkene present in **3**.

Significant differences in the ^{13}C NMR data obtained for **4** were that the resonances assigned to the ketal carbons C-10 and C-14 and the quaternary carbon C-13 in **1**, **2**, and **3** had been replaced by a ketone resonance at *δ* 214.2 (C-10), a tertiary carbinol resonance at *δ* 74.6 (C-14), and a methine resonance at *δ* 48.8 (C-13) in **4**, and the carbon resonance assigned to Me-15 had been shifted significantly downfield to *δ* 32.9 in **4** from ~*δ* 21 in **1**, **2**, and **3**. The ketone resonance (*δ* 214.2) was assigned to C-10 because it showed an HMBC correlation to the methyl singlet at *δ* 1.15, assigned to Me-25 (Figure 4). A proton resonance at *δ* 7.22 (s), which did not show an HSQC correlation to a carbon atom, was assigned to a tertiary alcohol at C-9. The OH-9 showed HMBC correlations to the ketone resonance at *δ* 214.2, assigned to C-10, a quaternary resonance at *δ* 65.5, assigned to C-8, an oxygenated methine resonance at *δ* 83.1, assigned to C-23, and a tertiary carbinol resonance at *δ* 89.5, assigned to C-9.

A second proton resonance at *δ* 5.22 (s), which did not show a HSQC correlation to a carbon atom, was assigned

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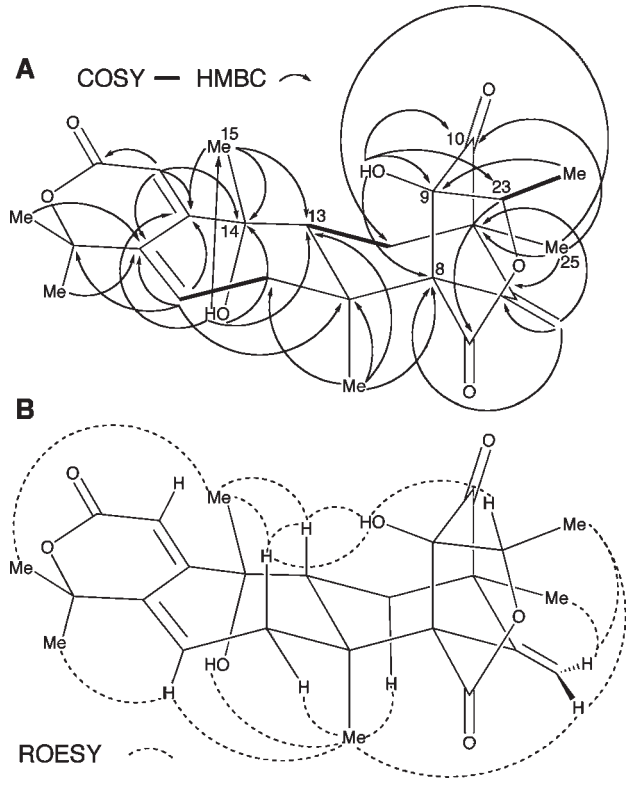

Figure 4. (A) COSY and HMBC correlartions observed for **4**; (B) ROESY correlations observed for **4**.

to a tertiary alcohol at C-14. The OH-14 resonance showed HMBC correlations to a tertiary carbinol resonance at δ 74.6, assigned to C-14, an olefinic resonance at δ 163.1, assigned to C-3, a methyl resonance at δ 32.9, assigned to Me-15, and a methine resonance at δ 48.8, assigned to C-13 (Figure 4). This set of OH-14 HMBC correlations required that there were carbon−carbon bonds between C-3 and C-14, C-14 and Me-15, and C-13 and C-14.

The proton resonance (δ 2.55 dd, J = 13.2, 5.4 Hz) assigned to the C-13 methine (δ 48.8) in the HSQC spectrum was shown in the COSY spectrum to be coupled to the methylene resonances assigned to H-12$_{eq}$/H-12$_{ax}$ (δ 1.87 dd, J = 13.2, 5.4 Hz/1.96 bt, J = 13.2 Hz), indicating a C-12/C-13 bond. Finally, an HMBC correlation observed between the methyl singlet at δ 1.08, assigned to Me-19, and the C-13 methine resonance at δ 48.8 required a carbon−carbon bond between C-7 and C-13 giving the seven-membered ring drawn in structure **4**.

The isodhilirane skeleton found in **4** can formally arise by 1,2 migration of the C-3 carbon from C-13 to C-14 accompanied by the conversion of the C-14/C-9 ether to an OH-9 in a hypothetical dhilirane intermediate that is related to **2** and **3**. Therefore, based on the assumption that the configurations at the common stereogenic centers in **1** and **4** are the same, a detailed analysis of the 2D ROESY data obtained for **4** (Figure 4) showed that the absolute configuration of **4** is 7*S*, 8*R*, 9*R*, 11*R*, 13*R*, 14*R*, 23*S*.

Phytopathogenic fungi are known to produce host selective phytotoxins that play a role in their pathogenesis.[4] With this in mind, the dhilirolides are being evaluated for possible toxic effects on *Averrhoa bilimbi* plants.

The new dhilirane and isodhilirane carbon skeletons have 25 carbon atoms. Simpson, Vederas, and Yamamura have shown that the 25 carbons in the fungal metabolites terretonin[5] and the citreohybridones[6] come from a mixed terpenoid/polyketide biogenesis involving farnesyl pyrophosphate and 3,5-dimethylorsellinate. It has been proposed that berkeleydione[7] and the miniolutelides[8] have the same meroterpenoid biogenetic origin.[9] Dhilirolide D (**4**) shares many structural features with berkeleydione and the miniolutelides. Therefore, it seems most likely that the dhilirolides are also highly rearranged meroterpenoids derived from farnesyl pyrophosphate and 3,5-dimethylorsellinate. Stable isotope feeding studies aimed at shedding light on the exact biogenesis of the new dhilirane (**5**) and isodhilirane (**6**) carbon skeletons are currently underway in our laboratories.

Acknowledgment. Financial support was provided by a grant from NSERC to R.J.A.

Supporting Information Available. Experimental details; tables of NMR data and NMR spectra for **1** to **4**; details of X-ray diffraction analysis of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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