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In Vivo Activity of Benzoyl Ester Clerodane Diterpenoid Derivatives from *Dodonaea polyandra*

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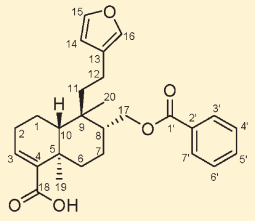
Supporting Information

ABSTRACT:



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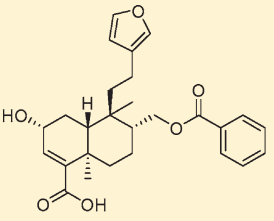

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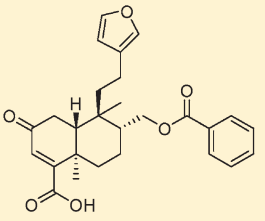

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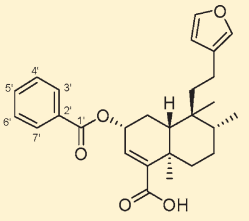

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Four new benzoyl ester clerodane diterpenoids, 15,16-epoxy-8α-(benzoyloxy)methylcleroda-3,13(16),14-trien-18-oic acid (**1**), 15,16-epoxy-8α-(benzoyloxy)methyl-2α-hydroxycleroda-3,13(16),14-trien-18-oic acid (**2**), 15,16-epoxy-8α-(benzoyloxy)methyl-2-oxocleroda-3,13(16),14-trien-18-oic acid (**3**), and 15,16-epoxy-2α-benzoyloxycleroda-3,13(16),14-trien-18-oic acid (**4**), have been isolated from the leaves and stems of *Dodonaea polyandra*. The anti-inflammatory activities of compounds **1**, **2**, and **4** were evaluated by means of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear edema. Compounds **2** and **4** exhibited maximum inhibition of inflammation (70−76%) at doses of 0.22 and 0.9 μmol/ear, respectively. Modest activity (∼45% inhibition) was maintained at nanomole/ear doses.

The plant genus *Dodonaea* (Sapindaceae) consists of around 68 species with 61 of these being endemic to Australia.[1] *Dodonaea polyandra* Merr. & L.M.Perry is described as a dioecious, erect shrub or small tree growing between 1.5 and 8.0 m in height.[1] In Australia, the distribution of *D. polyandra* is restricted to Cape York Peninsula, Queensland, particularly on the east and north coast and off-shore islands. It is also found in the western province of Papua New Guinea.

Our investigation of *D. polyandra* was undertaken as part of a collegial project initiated by traditional owners of the Northern Kaanju homelands (Kuuku I'yu) centered on the Wenlock and Pascoe Rivers in Cape York Peninsula in the state of Queensland, Australia. Traditional owners were keen to examine opportunities for development of their natural resources such as medicinal plants through partnerships with university-based researchers. *D. polyandra* is an important medicinal plant for relevant Kuuku I'yu Northern Kaanju traditional owners. The terminal end of the leaf (where leaf and stem meet) is generally placed in the cavity that remains after the removal of a rotten tooth to reduce inflammation of the localized area. More broadly, it may be used to relieve inflammation of the oral cavity. Testing of crude extracts of the species for anti-inflammatory activity in a 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced mouse ear edema model revealed that nonpolar extracts of the leaves had significant activity.[2]

Previously only one other *Dodonaea* species, *D. viscosa,* has been assessed for its anti-inflammatory properties based upon its use in traditional medicine in various countries.[3] Separate investigations have involved the screening of different extract types using the carrageenan-induced rat paw edema model.[4−6] However, none of these studies resulted in the identification or isolation of bioactive constituents that may explain the activity. The classes of secondary compounds most commonly isolated from *Dodonaea* species are flavonoids and clerodane diterpenoids. Other minor constituents include saponins, triterpenoids, steroids, and non-flavonoid phenyl propanoids.[3] However, these previous studies have not involved pharmacological testing of the isolated compounds.

This paper reports the isolation, elucidation, and anti-inflammatory activities of four new benzoyl ester clerodane diterpenoids (**1**−**4**) from *D. polyandra*. The results add to a Western scientific understanding of the medicinal actions of *D. polyandra,*

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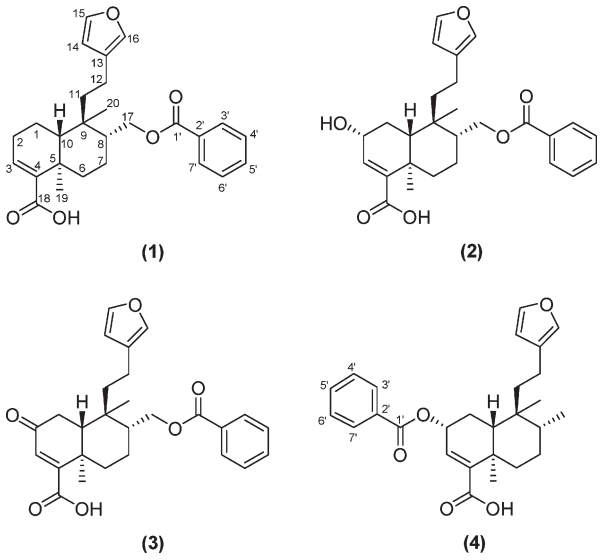
an important medicinal plant in Northern Kaanju traditional medicine.



RESULTS AND DISCUSSION

Bioactivity-guided fractionation of nonpolar extracts of the leaves of *D. polyandra* using solvent partitioning. Repeated column chromatography including normal phase and reverse phase C18, and normal phase HPLC led to the isolation of compounds **1**−**4**.

Clerodane diterpenoids are a structurally diverse and complex class of secondary metabolites.[7] As a consequence, a de novo determination of the structure of compound **1** will be considered first. Owing to the structural similarities of the isolated compounds, only differences relating to the remaining compounds (**2**−**4**) will be highlighted. Compound **1** was isolated as a white, amorphous solid. The molecular formula was determined to be $C_{27}H_{32}O_5$ by HREIMS with the molecular ion peak at m/z 436.2246 (calcd for $C_{27}H_{32}O_5$, 436.2250). This suggested that the compound contained 12 degrees of unsaturation. The IR spectrum displayed strong absorption bands for a hydroxy (3065 cm^{-1}), an ester carbonyl (1714 cm^{-1}), a conjugated carboxylic acid carbonyl (1681 cm^{-1}), an alkene (1630 cm^{-1}), and a furan (873 cm^{-1}) functionality. The ^{13}C NMR (Table 1) and DEPT spectra indicated the presence of 25 carbon resonances, two of which were methyl carbons (δ_C 20.5 and 19.2). Resonances for seven methylene carbons (δ_C 66.6, 38.9, 35.2, 27.4, 22.4, 18.3, and 17.0), two sp^3 methine carbons (δ_C 46.7 and 41.0), and nine sp^2 methine carbons (δ_C 142.8, 140.5, 138.6, 132.9, 129.5, 128.3, and 111.0) were observed (the signals at δ_C 129.5 and 128.3 were tentatively ascribed to two pairs of equivalent carbons on the basis of signal intensities). In total seven quaternary carbons were accounted for consisting of two carbonyl carbons (δ_C 171.1 and 166.7), three sp^2-hybridized carbons (δ_C 141.0, 130.4, and 125.1), and two sp^3 carbons (δ_C 38.4 and 37.4). Eight of the 12 degrees of unsaturation could be attributed to the presence of 14 sp^2 carbon centers (comprising two carbonyl groups and six carbon−carbon double bonds), suggesting that the remaining degrees of unsaturation were due to the existence of a tetracyclic ring structure.

The ^1H NMR spectrum of compound **1** showed resonances for five aromatic protons (δ_H 8.00, dd, J = 8.3, 1.3 Hz (2H); 7.54, tt, J = 7.3, 1.3 Hz; 7.42, dd, J = 8.3, 7.3 Hz (2H)), three furanyl protons (δ_H 7.33, t, J = 1.7 Hz; 7.19, dd, J = 1.7, 0.9 Hz; 6.27, dd, J = 1.7, 0.9 Hz), a vinylic proton (δ_H 6.89, dd, J = 4.8, 2.7 Hz), nonequivalent oxymethylene protons (δ_H 4.52, dd, J = 11.2, 4.5 Hz; 4.06, dd, 11.2, 8.0 Hz), two methyl singlets (δ_H 1.31,s; 0.92, s), and two methine signals (δ_H 1.96, m; 1.47, d, J = 12.6).

In the COSY spectrum, the aromatic proton at δ_H 8.00 (δ_C 129.5) coupled with the proton at δ_H 7.42 (δ_C 128.3), which further coupled to the proton at δ_H 7.54 (δ_C 132.9). Given the integration of signals for δ_H 8.00 and δ_H 7.42, this suggested the presence of a monosubstituted six-membered aromatic ring. The furanyl protons at δ_H 7.33 and δ_H 6.27 showed a COSY correlation. A third furan proton at δ_H 7.19 did not give COSY correlations with other furanyl protons. This indicated a β-substituted furan moiety within the structure.

HMBC correlations confirmed the substitution pattern of aromatic protons (δ_H 8.00, 7.54, and 7.42) (Figure 1), while the proton at δ_H 8.00 correlated with an sp^2 carbon (δ_C 166.7) characteristic of an ester carbonyl. In addition, the oxymethylene protons (δ_H 4.52 and 4.06) also correlated with C-1′, indicating compound **1** contained a benzoyl ester functionality.

The remaining resonances (δ_H 2.56, dt, J = 13.2, 3.4 Hz; 1.20, dt, 13.4, 3.6 Hz), (δ_H 2.47, ddd, J = 14.4, 12.2, 4.4 Hz; 2.25, m), (δ_H 2.36, dt, J = 19.6, 4.8 Hz; 2.20, m), (δ_H 1.83, m; 1.61, dq, J = 13.4, 3.4 Hz), (δ_H 1.73, m; 1.51, m), and (δ_H 1.78, m) were consistent with methylene protons of a fused cyclic ring system typical of clerodane diterpenoid compounds.[8,9]

The C-8 methine proton at δ_H 1.96 showed a COSY relationship with the oxymethylene protons (δ_H 4.52 and 4.06) and methylene protons at δ_H 1.83 and 1.61. The same methylene protons showed COSY correlations to H-6α and H-6β (attached to the carbon at δ_C 35.2). The methyl protons (δ_H 0.92) showed HMBC correlations to the methine carbon at δ_C 41.0 and a quaternary sp^3 carbon at δ_C 38.4. The second methyl group (δ_H 1.31) showed HMBC correlations to C-6, the quaternary sp^3 carbon (δ_C 37.4), and an sp^2-hybridized carbon (δ_C 141.0). The methine proton H-10 gave HMBC correlations with C-5 and a methyl carbon (δ_C 20.5). This suggested H-10 was positioned adjacent to the methyl group attached to C-5.

The vinylic proton (δ_H 6.89) showed COSY correlations with methylene protons (δ_H 2.36 and 2.20), and these also coupled with nonidentical methylene protons at δ_H 1.73 and 1.51. Furthermore, H-1β and H-1α coupled to H-10 in the COSY spectrum. An HMBC correlation was observed for H-2α and H-2β to the vinylic carbon (δ_C 141.0).

The remaining resonances in the ^1H and ^{13}C NMR spectra, which included methylene protons at δ_H 2.47 and 2.25 (δ_C 18.3), showed HMBC correlations to an sp^2 carbon (δ_C 125.1) and a furan sp^2 methine carbon (δ_C 138.6). Two equivalent methylene protons (δ_H 1.78) gave COSY correlations to nonequivalent methylene protons (δ_H 2.47 and 2.25) and HMBC correlation to a quaternary carbon at δ_C 38.4 to which the methyl group (δ_H 0.92, δ_C 19.2) was established to be attached. This confirmed the presence of a furanyl functionality containing an ethyl linkage between the β-carbon of the furan ring and quaternary carbon δ_C 38.4 (C-9). Further confirmation of this fragment was provided by the UV absorption at 225 nm, the IR absorption at 873 cm^{-1}, and the presence of significant ions at m/z 81 (C_5H_5O) and 95 (C_6H_7O) in the EIMS. These cleavages in the mass spectrum are commonly seen in clerodane diterpenoids containing a furanyl group with an ethyl side chain.[10]

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Table 1. NMR Spectroscopic Data (600 MHz) for Compounds 1−4

	1[a]		2[a]		3[a]		4[a]	
pos	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)
1	17.0, CH_2	β 1.73, m; α 1.51, m	26.8, CH_2	β 1.96, m; α 1.76, m	34.6, CH_2	2.51, d (8.8)	24.8, CH_2	β 1.95, ddd (15.0 12.8, 5.0); α 2.02, dq (15.0, 1.8)
2	27.4, CH_2	α 2.36, dt (19.6, 4.8); β 2.20, m	66.2, CH	4.39, dt (4.5, 1.4)	200.2, C		67.6, CH	5.65, dt (4.8, 1.4)
3	140.5, CH	6.89, dd (4.8, 2.7)	136.5, CH	6.72, dd (4.5, 1.4)	130.2, CH	6.41, bs	132.7, CH	6.77, dd (4.4, 1.4)
4	141.0, C		143.9, C		142.9, C		146.4, C	
5	37.4, C		38.0, C		38.5, C		38.0, C	
6	35.2, CH_2	α 2.56, dt (13.2, 3.4); β 1.20, dt (13.4, 3.6)	34.7, CH_2	α 2.46, m; β 1.25, dt (13.2, 3.6)	34.2, CH_2	α 2.38, dt (12.6, 3.0); β 1.44, m	35.3, CH_2	α 2.36, m; β 1.30, dt (12.6, 4.1)
7	22.4, CH_2	β 1.83, m; α 1.61, dq (13.4, 3.6)	22.3, CH_2	β 1.85, m; α 1.63, dq (13.2, 3.6)	21.8, CH_2	β 1.93, m; α 1.71,m	27.1, CH_2	β 1.48, m; α 1.51, m
8	41.0, CH	1.96, m	40.9, CH	2.01, m	40.7, CH	2.01, m	36.1, CH	1.64, m
9	38.4, C		38.1, C		38.3, C		38.5, C	
10	46.7, CH	1.47, d (12.6)	41.4, CH	1.75, m	45.6, CH	2.09, t (8.8)	42.1, CH	1.78, dd (12.8, 2.0)
11	38.9, CH_2	1.78, m	38.9, CH_2	1.77, m	38.3, CH_2	1.92, m; 1.71, m	38.3, CH_2	1.57, m
12	18.3, CH_2	2.47, ddd (14.4, 12.2, 4.8); 2.25, m	17.8, CH_2	2.56, m; 2.44, m	18.1, CH_2	2.49, m; 2.23, m	17.7, CH_2	2.38, m; 2.30, m
13	125.1, C		125.2, C		124.3, C		125.0, C	
14	111.0, CH	6.27, dd (1.7, 0.9)	111.1, CH	6.28, dd (1.7, 0.9)	110.8, CH	6.25, dd (1.7, 0.9)	110.7, CH	6.00, dd (1.7, 0.9)
15	142.8, CH	7.33, t (1.7)	142.6, CH	7.31, t (1.7)	142.9, CH	7.32, t (1.7)	142.5, CH	7.20, t (1.7)
16	138.6, CH	7.19, dd (1.7, 0.9)	138.6, CH	7.19, dd (1.7, 0.9)	138.7, CH	7.18, bs	138.3, CH	6.76, dd (1.7, 0.9)
17	66.6, CH_2	4.52, dd (11.2, 4.5); 4.06, dd (11.2, 8.0)	66.4, CH_2	4.53, dd (11.1, 4.6); 4.08, dd (11.1, 7.8)	65.9, CH_2	4.54, dd (11.4, 4.8); 4.08, dd (11.4, 8.4)	15.8, CH_3	0.86, d (6.6)
18	171.1, C		171.0, C		168.5, C		171.1, C	
19	20.5, CH_3	1.31, s	18.8, CH_3	1.27, s	18.7, CH_3	1.45, s	18.9, CH_3	1.29, s
20	19.2, CH_3	0.92, s	19.2, CH_3	0.92, s	18.7, CH_3	1.10, s	18.0, CH_3	0.79, s
1′	166.7, C		166.7, C		166.6, C		165.9, C	
2′	130.4, C		130.3, C		130.1, C		129.9, C	
3′	129.5, CH	8.00, dd (8.3, 1.3)	129.5, CH	8.00, dd (8.3, 1.4)	129.5, CH	8.00, dd (8.3, 1.3)	129.7, CH	8.01, dd (8.4, 1.3)
4′	128.3, CH	7.42, dd (8.3, 7.3)	128.4, CH	7.41, dd (8.3, 7.4)	128.4, CH	7.42, dd (8.3, 7.4)	128.4, CH	7.37, dd (8.4, 7.4)
5′	132.9, CH	7.54, tt (7.3, 1.3)	132.9, CH	7.55, tt (7.4, 1.4)	133.0, CH	7.56, tt (7.4, 1.3)	133.2, CH	7.53, tt (7.4, 1.3)
6′	128.3, CH	7.42, dd (8.3, 7.3)	128.4, CH	7.41, dd (8.3, 7.4)	128.4, CH	7.42, dd (8.3, 7.4)	128.4, CH	7.37, dd (8.4, 7.4)
7′	129.5, CH	8.00, dd (8.3, 1.3)	129.5, CH	8.00, dd (8.3, 1.4)	129.5, CH	8.00, dd (8.3, 1.3)	129.7, CH	8.01, dd (8.4, 1.3)

[a] $CDCl_3$ was used as solvent; no signals for OH observed in ^1H NMR.

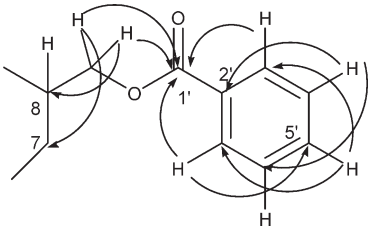

Figure 1. HMBC correlations of the benzoyl ester moiety of compound **1**.

A substructure with a molecular mass of 391 amu was obtained having exhausted the available data from NMR, meaning there remained an unaccounted mass of 45 amu. This indicated the presence of a carboxylic acid group, which was supported by a peak in the ^{13}C NMR spectrum at δ_C 171.1 as well as an −OH stretch at 3065 cm^{-1} in the IR spectrum. On the basis of the three-bond HMBC correlations of the vinylic proton (δ_H 6.77) to the carbon at δ_C 171.1, the carboxylic acid was located at the quaternary carbon resonating at δ_C 141.0. NMR data for similar compounds further supported this positioning.[8,11] The structure of this new compound was established to be the clerodane furanoditerpenoid **1**, 15,16-epoxy-8α-(benzoyloxy)methylcleroda-3,13(16)14-trien-18-oic acid (systematic name: *rel*-(4a*R*,5*S*,6*S*,8a*S*)-6-[(benzoyloxy)methyl]-5-[2-(3-furyl)ethyl]-5,8a-dimethyl-3,4,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylic acid).

The assignment of the relative configuration of the stereocenters of **1** was carried out using ROESY data as well as information obtained from previously isolated clerodane diterpenoids from *Dodonaea* spp.[11−13] Clerodane diterpenoids previously isolated from the genus *Dodonaea* having a C-3−C-4 double bond usually contain a *trans* arrangement at the ring junction.[3,11] The Me-19 may also serve as a basis for determining the conformation of the A/B ring junction; for *trans* fusion the chemical shift of the C-19 methyl is typically in the range δ_C 11−19, in contrast to *cis* fusion, where the C-19 methyl resonance is observed to be δ 5−10 higher, typically δ_C 25.[14] In this case, the C-19 methyl resonance is observed at δ_C 20.5, which is on the

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lower end of the chemical shift range, which may indicate a *cis* orientation (although the shift is slightly out of the usually observed range). However, the Me-19 resonance of a structurally related compound, (−)-hardwickiic acid, for which the X-ray crystal structure has been determined, also displays a slightly higher chemical shift for the *trans* Me-19 (δ 20.6).[9,15] In addition the chemical shift of Me-20 (δ 19.2), which is typical of *trans* clerodanes,[14] supported a *trans* ring junction in **1**. From ROESY data the C-19 methyl group (δ_H 1.31) showed correlation with the C-20 methyl (δ_H 0.92), which in turn showed correlation with the oxymethylene protons of C-17 (δ_H 4.52, 4.06). These observations, together with other ROESY correlations, established the relative configurations for carbons C-1, C-5, C-8, and C-9 as shown in Figure 2.

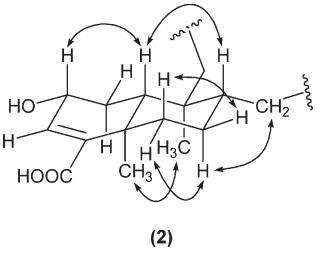
It was revealed from analysis of the NMR spectroscopic data of compound **2** that this compound was also a clerodane furanoditerpenoid with distinct similarities to that of compound **1**. The molecular formula was determined to be $C_{27}H_{32}O_6$ from the M − H peak at *m/z* 451.2123 (calcd for $C_{27}H_{31}O_6$, 451.2121). The most notable difference in the ^{1}H NMR spectrum of compound **2** was an oxymethine proton (δ_H 4.39, dt, *J* = 4.5, 1.4 Hz), which showed strong COSY correlations with the vinylic proton (δ_H 6.27, dd, *J* = 4.5, 1.4 Hz), which was established to be in the same position at C-3 (δ_C 136.5) as seen in compound **1**. This indicated the presence of a hydroxy functionality at this position in comparison to **1**. Although the existence of a hydroxy group was not apparent from the ^{1}H NMR or IR spectrum, the total number of carbons and MS accurate mass strongly supported this proposition. The oxymethine proton H-2 also showed COSY correlation with chemically nonequivalent methylene protons (δ_H 1.96, m and 1.76, m) at position C-1 (δ_C 26.8). An unassigned carboxylic acid carbon (δ_C 171.0) was also attached to the C-4 vinylic carbon (δ_C 143.9) as determined for compound **1** on the basis of HMBC correlations between the vinylic proton at C-3 and carbonyl carbon C-18. The structure of this new compound was established to be the clerodane diterpenoid **2**, 15,16-epoxy-8α-(benzoyloxy)methyl-2α-hydroxycleroda-3,13(16),14-trien-18-oic acid (systematic name *rel*-(3*R*,4a*R*,5*S*,6*S*,8a*S*)-6-[(benzoyloxy)methyl]-5-[2-(3-furyl)ethyl]-3-hydroxy-5,8a-dimethyl-3,4,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylic acid).

The assignment of the relative configuration at C-10 in compound **2** was assumed to be the same as that of **1**. A ROESY experiment permitted establishment of the relative configurations for C-2, C-5, C-8, C-9, and C-10 as shown in Figure 3.

Compound **3** was isolated as an off-white, amorphous solid with the molecular formula $C_{27}H_{30}O_6$, which was determined from the molecular ion $[M]^+$ peak at *m/z* 450.2041 (calcd for $C_{27}H_{30}O_6$, 450.2042). A two-proton difference between this compound and **2** indicated the presence of an additional degree of unsaturation. The ^{1}H NMR spectrum of compound **3** was similar to that of **2** with one exception being the absence of the oxymethine proton that was observed at δ_H 4.39 in the ^{1}H NMR spectrum of compound **2**. The ^{13}C NMR spectrum showed distinct similarities to that of compound **2**; however a significant peak at δ_C 200.2, which was not present in the spectrum of **2**, suggested that a ketocarbonyl functional group was present in compound **3** instead of the secondary alcohol observed in **2**. The position of this group was determined to be at C-2, as indicated by HMBC correlations from the C-1 methylene protons (δ_H 2.51) and H-10 (δ_H 2.09) to the carbon at δ_C 200.2. Given the minor structural differences described above, the structure of this new compound **3** was established as 15,16-epoxy-8α-(benzoyloxy)methyl-2-oxocleroda-3,13(16),14-trien-18-oic acid (systematic name *rel*-(4a*R*,5*S*,6*S*,8a*S*)-6-[(benzoyloxy)methyl]-5-[2-(3-furyl)ethyl]-5,8a-dimethyl-3-oxo-3,4,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylic acid).

The assignment of relative configuration to stereocenters used similar evidence to that previously described. Both methyl groups (δ_H 1.45, δ_C 18.7 and δ_H 1.10, δ_C 18.7) showed ROESY correlations; however neither correlated with H-10, again suggesting a *trans* configuration at the ring junction. H-10 and H-8 showed a ROESY correlation indicating that the benzoyl ester moiety was orientated in the same position as for **1** and **2**. This established the relative configurations of C-5, C-8, C-9, and C-10 as being the same in **1** and **2**.

Compound **4** was isolated as a white, amorphous solid with the molecular formula $C_{27}H_{32}O_5$, which was determined from the HREIMS molecular ion peak at *m/z* 436.2245 (calcd for $C_{27}H_{32}O_5$, 436.2250). The ^{1}H NMR spectrum for compound **4** showed similarities to the spectra of **1** and **2**, with the notable absence of the signal pertaining to oxymethylene protons within the 4.00−4.50 ppm range. A vinylic proton at δ_H 6.77, which initially showed up as a doublet, was resolved into a double doublet upon Lorentzian/Gaussian resolution enhancement[16] (dd, *J* = 4.4, 1.4 Hz; δ_C 132.7) and gave a strong COSY correlation with an oxymethine proton (δ_H 5.65, dt, *J* = 4.8, 1.4 Hz). In the HMBC spectrum this same proton (H-2) correlated with the ester carbonyl carbon at δ_C 165.9. Given the commonality of a C-3 vinylic proton in clerodane diterpenoids, this meant the vinylic



(2)

Figure 3. Selected ROESY correlations for **2**.

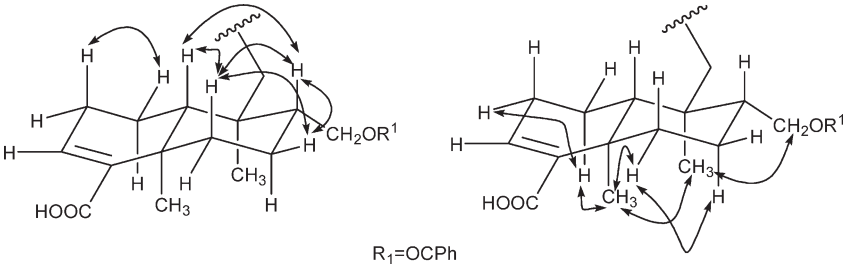


Figure 2. Selected ROESY correlations for **1**.

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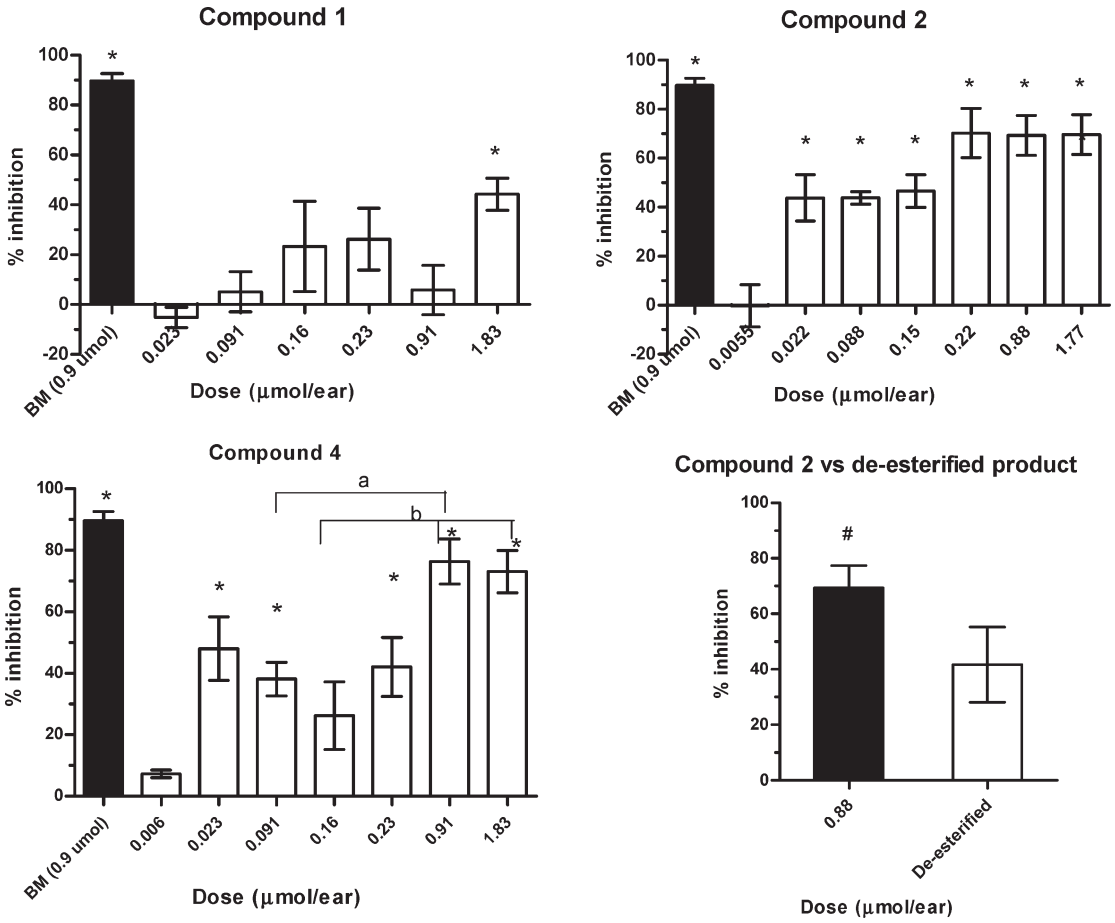

Figure 4. Graphical representation of dose−response characteristics of isolated compounds **1**, **2**, and **4** and comparison of the activities between **2** and its de-esterified product in a TPA-induced mouse ear edema model of inflammation. Doses presented are μmol/ear. Percentage inhibition data are presented as the mean ± SEM (for **1** $n = 4$, for **2** and **4** $n \geq 6$ for each treatment group, data calculated at $t = 8$ h); $^{*}p < 0.05$ compared to negative TPA control; $^{a,b}p < 0.05$, a statistically significant difference exists between treated groups of the same compound; $^{\#}p < 0.05$, a statistically significant difference exists between compound **2** and its de-esterified product at a dose of 0.88 μmol/ear. BM = betamethasone-17,21-dipropionate.

proton was located at C-3 and implied the benzoyl ester functionality was attached at C-2, unlike in compounds **1**, **2**, and **3**, where it was at C-8. This structural feature also lacked the nonidentical oxymethylene protons that were associated with compounds **1** and **2** (δ_H 4.52, 4.06/δ_H 4.53, 4.08), further supporting this.

In contrast to the structures of **1**−**3**, evidence for a third methyl group was seen in the DEPT and ^{1}H NMR data. A methyl doublet (δ_H 0.86, d, $J = 6.6$ Hz) coupled with a methine proton (δ_H 1.64, m), which also showed COSY correlations with the methylene protons at δ_H 1.51 and 1.48, thus indicating that the methyl group was positioned at C-8 (δ_C 36.1). The connectivity for the methyl group (δ_H 0.79) that appeared as a singlet in the ^{1}H NMR spectrum was determined to be connected at δ_C 38.5 (C-9) through HMBC correlations with C-8, C-9, and C-10.

These structural data led to the new compound **4** being 15,16-epoxy-2α-benzoyloxycleroda-3,13(16),14-trien-18-oic acid (systematic name *rel*-(3*R*,4a*R*,5*R*,6*S*,8a*S*)-3-(benzoyloxy)-5-[2-(3-furyl)ethyl]-5,6,8a-trimethyl-3,4,4a,5,6,7,8,8a-octahydronaphthalene-1-carboxylic acid). The relative configurations of stereocenters in **4** were established to be the same as in **1** and **2**.

Compounds **1**, **2**, and **4** showed significant anti-inflammatory activity in a TPA-induced mouse ear edema model, with the most potent being **2** and **4** (the quantity of **3** did not allow for testing). While compound **1** showed a significant level of activity at the highest dose tested (1.83 μmol/ear, $p < 0.05$) compared to the nontreated (TPA) control, there was no observable effect at lower concentrations. Compound **2** showed a dose−response over the dose range 0.0055−1.77 μmol/ear. At 8 h post-application, 0.22 μmol/ear dose gave a maximum of 70.2 ± 10.0% (mean ± SEM) inhibition, with no improvement in activity observed for the two higher doses. Compound **4** showed equally potent activity as **2**, with maximum inhibition of 76.4 ± 7.3% achieved at a dose of 0.91 μmol, with activities comparable to the positive control betamethasone dipropionate (0.90 μmol/ear). However, the dose−response relationship of **4** was characterized by a U-shaped dose−response over the experimental range, featuring significant activities at low and high doses. The shape of this dose−response is actually rather common of immune system-related end points; however the significance of such phenomena is often ignored.[17] While a number of classes of compounds have been reported to induce such dose−responses, steroids are noteworthy examples typically associated with having these effects.[17,18] A further point of interest is that BALB/c mouse models are commonly associated with displaying these types of dose−responses.[17] The mechanistic basis for these types of dose−responses is complex and multivariate. Further information regarding the mechanism of action of compounds **1**, **2**, and **4** is required in order to explain the observed responses.

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In the broadest sense, diterpenoid secondary compounds are well documented for their ability to exert anti-inflammatory effects both in vivo and in vitro.[19] However, a more specific focus on the anti-inflammatory properties of the clerodane class of diterpenoid reveals rare occurrences of such activity, with only a couple of investigations reporting anti-inflammatory activities of clerodane-type furanoditerpenoids.[20,21]

To determine whether the benzoyl ester moiety was important for the observed activity, compound **2** was subjected to mild alkaline hydrolysis[22] and subsequently tested in vivo. As shown in Figure 4, removal of this functional group significantly reduced ($p < 0.05$) the ability of the compound to inhibit inflammation (edema) in vivo. This result does not clarify whether the benzoyl ester is specifically involved in interactions between ligand and target or whether it may play a role in improving bioavailability. For example, the increased lipophilicity imparted by the group suggests that it may be important for absorption into the skin.

The most active compounds, **2** and **4**, are currently under investigation for potential development as inhibitors of skin inflammatory diseases. Mechanism of action studies involving in silico and in vitro techniques are being investigated.

In conclusion, this study revealed for the first time four new benzoyl ester clerodane diterpenoid derivatives isolated from *D. polyandra*. The anti-inflammatory activities of these compounds have been evaluated and contribute to a Western scientific understanding of the traditional use of the plant in Northern Kaanju (Kuuku I'yu) medicine. Their potential as new anti-inflammatory agents is currently being explored.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting point determination was carried out on a Stuart SMP10 melting point apparatus (Bibby Scientific Ltd., Staffordshire) and is uncorrected. Optical rotations were conducted using a PolAAr 21 polarimeter (589 nm, 20 °C). UV and IR spectroscopy were carried out using a CARY 50 Bio (Varian, USA) and Shimadzu 8400S FT-IR (Shimadzu, Japan), respectively. The 1D and 2D NMR data were acquired on a Varian INOVA 600 MHz spectrometer ($CDCl_3$ or methanol-d_4) at the University of Adelaide. The HREIMS and/or HRAPCI mass spectra were obtained on a Kratos Concept ISQ magnetic sector or ThermoFinnigan LTQ Orbitrap HR MS/MS at the Central Science Laboratory, University of Tasmania. All solvents used for plant extraction, crude separations, or synthesis were analytical grade (Merck, Australia and AJAX Chemicals, Australia) or HPLC grade (Merck, Australia and Univar, Australia) for HPLC experiments. TLC plates (reverse phase RP-18 F_{254} and normal phase silica gel 60 F_{254}) were purchased from Merck (Darmstadt, Germany). Plates were visualized under UV light (254/365 nm) using a Chromatovue cabinet CC-60 (UVP, Australia). Anisaldehyde reagent was prepared as described previously.[2] Waters C18 125 Å (Milford, MA), Merck silica gel 60 (70−230 mesh ASTM), and Sephadex LH-20 (Sigma) were used for column chromatography. All HPLC experiments were carried out on a Shimadzu SIL-10A with auto injector, SCL-10A system, with Activon GoldPak 100 5 μm ODS 25 × 1 cm or Activon GoldPak silica gel 5 μm semipreparative HPLC columns.

Plant Material. Plant material was collected by Kaanju people at Chuula outstation (Kaanju homelands), Central Cape York Peninsula, Queensland, in collaboration with ethnobotanist Mr. Nick Smith, who confirmed Western scientific species name. The leaves and stems of *D. polyandra* were collected from a population of 30 plant samples from both male and female types (juveniles ≤2 m) in December 2007. Voucher specimens and details of plant location (13°07′14″, 142°59′45″) were recorded and lodged at Brisbane Herbarium, Queensland (voucher specimen number AQ 749703). Plant material was allowed to air-dry in the shade and packed into paper bags for transportation. Leaves and stems were separated and stored in separate paper bags at −20 °C until extraction.

Extraction and Isolation. A sequential extraction of *D. polyandra* leaves (500 g) was performed with *n*-hexane (SeLH), DCM/MeOH (1:1) (SeLMM), and 80% aqueous EtOH (SeLE) using a solvent to dry plant material in a ratio of 5:1 at 25 °C with agitation for 24 ± 1 h for each solvent. After 24 h the respective extracts were decanted, filtered, and dried in vacuo. The respective mass recovery yields for the SeLH, SeLMM, and SeLE extracts were 1.4%, 15.4%, and 14.1%.

The SeLH extract (7 g) was initially separated by normal phase flash chromatography on silica gel using a mobile phase of *n*-hexane with increasing amounts of DCM up to 100% DCM, to which MeOH was introduced beginning with a 99:1 ratio. The separation was ceased once the column had been flushed with DCM/MeOH (90:10). Alternate fractions were analyzed by TLC and pooled into larger fractions based on the observed profile. A total of 13 main fractions (LH1 to 13) were obtained, with each subsequently tested in the TPA-induced mouse ear edema model. Fractions displaying significant activity were further fractionated, using the process described below. TLC profiles were used to examine the pattern of significant components present in each of the active fractions, and the separation procedures designed to isolate these components.

Fraction (Fr) LH11 (1132 mg) was separated into five subfractions using low-pressure reverse phase (C18) column chromatography and isocratic elution with 90% MeOH/H_2O. The separation was continuously monitored by TLC analysis. The major bands (on TLC) of the fraction stained green-blue in appearance after spraying with anisaldehyde reagent. Remaining components were flushed off with 100% MeOH followed by 2-propanol. The bulk of the separation was contained within Fr-LH11.3 (606 mg). This fraction (75 mg) was further purified using normal phase HPLC with *n*-hexane/EtOAc (8:2) isocratic elution to yield 44 mg of compound **4** as a white, amorphous solid.

Further separation of Fr-LH9 (495 mg) was conducted in a similar manner to Fr-LH11 using low-pressure reverse phase column chromatography. The fraction also contained a major green-blue band on TLC with an R_f different from that of compound **4**. The reverse phase column separation gave three fractions (LH9.1, LH9.2, and LH9.3), with Fr-LH9.2 containing the component that stained green-blue. A portion of this fraction (65 mg) was repeatedly washed with cold *n*-hexane/EtOAc (8:2) to yield compound **1** (40 mg) as a white, amorphous solid.

An initial cleanup of the SeLMM extract (15 g) was undertaken by successive solvent−solvent partioning between 70% MeOH/H_2O with *n*-hexane and DCM. The DCM fraction Fr-SeLMM2 (7.00 g) contained the component of interest. This fraction (2.24 g) was subjected to repeated low-pressure reverse phase (C18) column chromatography under isocratic elution with 85% MeOH/H_2O. The separation was monitored by normal phase TLC until the band of interest was obtained. Five fractions (SeLMM2.1−SeLMM2.5) were pooled together on the basis of TLC analysis, with Fr-SeLMM2.4 containing the blue-green spot. The SeLMM2.4 fraction (255 mg) was purified further by repeated low-pressure normal phase column chromatography with DCM/MeOH (99:1) as the mobile phase to yield compound **2** (50 mg) as a white, amorphous solid. Crystallization of **2** was achieved using a secondary container technique with sample dissolved in EtOAc and *n*-hexane used as the diffusing solvent.

An extraction of *D. polyandra* stems with DCM/MeOH (1:1) was prepared using the same procedure as described above for leaf extract. The yield of extract (DPS) was 34.9 g (9.8%). The extract was further purified by liquid/liquid partitioning between DCM and 70% aqueous MeOH. The DCM fraction (DPS1) was dried in vacuo, giving a yield of 14.4 g. An initial separation of 10.1 g of fraction DPS1 was carried out using normal phase column chromatography eluting with *n*-hexane/

DCM with increasing amounts of DCM, ceasing with DCM/MeOH (95:5). Fractions were grouped into three major fractions based on the TLC profiles.

The first fraction, DPS1A (4.1 g), was separated under reverse phase conditions eluting with 75% aqueous MeOH with increasing amounts of MeOH to afford three major fractions (DPS1A1, DPS1A2, and DPS1A3). Fraction DPS1A1 (685 mg) was passed through a Sephadex LH-20 column eluting with DCM/MeOH (3:1), giving two fractions (DPS1A1a and DPS1A1b). Fraction DPS1A1a (40 mg) was separated using normal phase preparative TLC (DCM/MeOH, 95:5) to yield 1 mg of compound **3** as an off-white solid.

Mouse Ear Edema Assay. Anti-inflammatory activity was measured by a well-characterized mouse ear edema model using 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Sigma) as the inducer of inflammation as described previously.[2] Briefly, baseline measurements of ear thickness were measured using a digital micrometer (±0.001 mm, Mitutoyo, Japan) prior to the experiment. 12-*O*-Tetradecanoylphorbol-13-acetate (2.5 μg/ear) (dissolved in acetone) was then applied in a volume of 20 μL to the inner surface of the right ear and 20 μL of acetone to the left ear as control. Thirty minutes later the test samples (dissolved in 80% aqueous EtOH) were applied to the inner surface of the right ear and 80% aqueous EtOH on the left ear to the respective treatment groups. Betamethasone-17,21-dipropionate (Sigma) was used as a positive control (0.9 μmol/ear). Fractions were tested as previously described.[2] Multidose experiments were conducted over the range 0.005−1.83 μmol/ear for isolated compounds. At 2, 4, 6, 8, 24, and 48 h after the application, the ear thicknesses were measured using a digital micrometer. A nontreated control group to which only TPA was applied was used as a measure of maximum inflammation achieved, with percent inhibition of inflammation of test sample being calculated relative to this group. Following completion of the experiment mice were euthanized by inhalation of isofluorane followed by cervical dislocation. Experiments were conducted in accordance with ethical standards as outlined in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (9th ed.) and approved by The Institute of Medical and Veterinary Science Animal Ethics Committee.

Alkaline Hydrolysis. Mild alkaline ester hydrolysis of compound **2** was carried out using a previously described method.[22] Briefly, KOH (Merck, Darmstadt) was reacted with the compound using a mole ratio of 3:1 in 100 μL of MeOH at 37 °C. The progress of the reaction was monitored and terminated at 2 h by addition of 300 μL of H_2O, followed by 100 μL of 1 M HCl. The component of interest was recovered by liquid−liquid extraction with 500 μL of DCM, and the organic layer was subsequently removed from the aqueous layer, dried over Na_2SO_4, and centrifuged (14 000 rpm for 5 min) to remove solid particulates. The dried organic layer containing the hydrolyzed component of interest was purified by preparative TLC under normal phase conditions (92.5:7.5 DCM/MeOH). The structure of the hydrolysis product was confirmed by NMR.

Statistical Analysis. Calculations, statistics, and graphing were carried out using Microsoft Excel, SPSS (version 17.0) and GraphPad (version 5.00). Maximum percent inhibitions were determined at the 8 h time point by calculating the response of test samples relative to negative control. Statistical significance was determined using one-way ANOVA or independent *t* test (where applicable). The result was deemed significant when $p < 0.05$.

15,16-Epoxy-8α-(benzoyloxy)methylcleroda-3,13(16),14-trien-18-oic acid (1): white, amorphous solid; $[\alpha]^{20}_D$ −69.5 (*c* 0.81, MeOH); UV (MeOH) λ_{max} (log ε) 225 (4.28) and 275 (3.06) nm; IR ($CHCl_3$) ν_{max} 3065, 1714, 1681, 1630, 1602, 1276, and 873 cm^{-1}; ^1H and ^{13}C NMR data shown in Table 1; LREIMS *m/z* 436 $[M]^+$ (5), 219 (100), 125 (50), 105 (70), 95 (55), and 81 (45); HREIMS *m/z* 436.2246 (calcd for $C_{27}H_{32}O_5$, 436.2250).

15,16-Epoxy-8α-(benzoyloxy)methyl-2α-hydroxycleroda-3,13(16),14-trien-18-oic acid (2): white, crystalline needles (*n*-hexane/EtOAc); mp 81−83 °C; $[\alpha]^{20}_D$ −75.4 (*c* 0.84, MeOH); UV (MeOH) λ_{max} (log ε) 225 (4.22), 270 (3.08), and 345 (2.67) nm; IR ($CHCl_3$) ν_{max} 3090, 1712, 1692, 1633, 1602, 1278, and 873 cm^{-1}; ^1H and ^{13}C NMR data shown in Table 1; LRAPCI *m/z* 451 [M − H]; HRAPCI *m/z* 451.2123 (calcd for $C_{27}H_{31}O_6$, 451.2121).

15,16-Epoxy-8α-(benzoyloxy)methyl-2-oxocleroda-3,13(16),14-trien-18-oic acid (3): off-white solid; $[\alpha]^{20}_D$, UV, and IR not determined due to insufficient material; ^1H and ^{13}C NMR data shown in Table 1; LREIMS *m/z* 450 $[M]^+$ (5), 417 (10), 234 (10), 189 (12), 105 (60), 95 (100), 81 (32), 77 (28); HREIMS *m/z* 450.2041 (calcd for $C_{27}H_{30}O_6$, 450.2042).

15,16-Epoxy-2α-benzoyloxycleroda-3,13(16),14-trien-18-oic acid (4): white, amorphous solid; $[\alpha]^{25}_D$ −199.2 (*c* 0.46, MeOH); UV (MeOH) λ_{max} (log ε) 225 (4.35), 275 (3.06), and 345 (2.62) nm; IR ($CHCl_3$) ν_{max} 3510, 1708, 1693, 1635, 1600, and 873 cm^{-1}; ^1H and ^{13}C NMR data shown in Table 1; LREIMS *m/z* 436 $[M]^+$ (5), 299 (48), 122 (50), 105 (100), and 81 (50); HREIMS *m/z* 436.2245 (calcd for $C_{27}H_{32}O_5$, 436.2250).

■ ASSOCIATED CONTENT

Supporting Information. NMR and MS spectra for compounds **1** to **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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