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PHEBALOPARVILACTONE: A NEW PROTOLIMONOID FROM THE LEAVES OF *PHEBALIUM SQUAMULOSUM* SSP. *PARVIFOLIUM*

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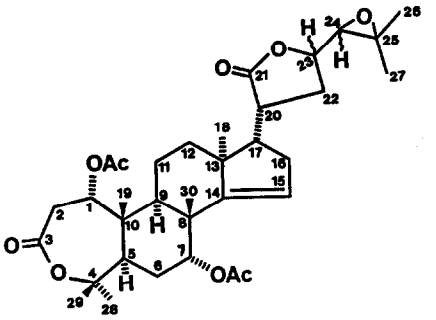
Abstract: Phebaloparvilactone (1), isolated from the aerial parts of *Phebalium squamulosum* ssp. *parvifolium* (Rutaceae), has been characterized, primarily on the basis of detailed NMR studies, as the new protolimonoid 1α,7α-diacetoxy-3,21-oxo-3,4-*seco*-apotirucalla-14-en-3,4;21,23;24,25-trioxide.

The plant family Rutaceae is a rich source of alkaloids, coumarins, flavonoids and limonoids.[1] We have recently undertaken the investigation of several species of the Australian genus *Phebalium* from which we have isolated a wide range of coumarins.[2-5] In the course of these studies we have obtained, from the petrol extract of the aerial parts of *Phebalium squamulosum* ssp. *parvifolium* P. G. Wilson, a new protolimonoid. This compound, to which we have assigned the trivial name phebaloparvilactone, is the first limonoid type triterpene to be isolated from *Phebalium*. In the identification of phebaloparvilactone as (1) extensive use has been made of a wide range of NMR techniques and these are discussed below.

Vacuum liquid chromatography of a concentrate of the combined petrol and ethyl acetate extracts of the powdered leaves of *Ph. squamulosum* ssp. *parviflorum* over TLC grade silica gel, followed by preparative TLC, yielded five pure compounds of which four were identified as the coumarins aurapten, umbelliferone, 7′-hydroxy-3′,7′-dimethyl-2′,5′-octadienyloxycoumarin and scopoletin.



(1)

Phebaloparvilactone (1) was obtained as an amorphous solid in a total yield of approximately 5 mg. All analyses were performed on this sample. On TLC (silica gel) it gave a single purple spot when sprayed with vanillin/H_2SO_4 followed by heating to 100°C. The IR spectrum showed absorption bands at 1775 and 1731 cm^{-1} indicative of several carbonyl functions. The EIMS revealed a fragment at m/z 480, which analysed for $C_{30}H_{40}O_5$. However, the FAB-MS showed the pseudo-molecular ion at m/z 601 $[M+H]^+$, which suggested an empirical formula $C_{34}H_{48}O_9$.

The ^1H NMR spectrum was run at 300 MHz in $CDCl_3$ and correlated with the ^{13}C chemical shifts by means of a Heteronuclear Multiple Quantum Coherence (HMQC) experiment through which direct C-H couplings were revealed (Table 1). HMQC and HMBC (see later) are inverse heteronuclear correlation techniques in which ^{13}C pulses are sent through the transmitter and ^1H pulses through the decoupler. These are much more sensitive than the corresponding HETCOR and COLOC procedures and were essential to the study in view of the small amount of material available (see Experimental for more details). The ^1H NMR spectrum showed several notable features including seven methyl singlets (δ 1.01, 1.16, 1.18, 1.35, 1.37, 1.40 and 1.51), two acetoxyls (δ 2.00, 2.10), one olefinic proton (δ 5.30), three oxymethine resonances (δ 5.17, 4.83, 4.18) and an epoxy oxymethine (δ 2.81).[6] The ^{13}C NMR spectrum indicated the carbonyls (δ_C 170.4, double intensity) and this was confirmed by FT-IR (1775, 1731 cm^{-1}).

Table 1. ^{1}H and ^{13}C NMR chemical shift and ^{1}H coupling constant data for phebaloparvilactone (1)

C/H	δ_C	δ_H	H multiplicity	$^{2}J_{H-H}$	$^{3}J_{H-H}$
1	70.9	4.83	t		4.1
2	34.8	3.16	d		4.1
3	170.4	-			
4	85.5	-			
5	44.1	2.52	dd		12.2, 3.3
6	26.2	2.00-1.85	m		
7	74.3	5.17	t		2.9
8	41.9	-			
9	35.7	2.59	dd		11.0, 5.0
10	44.2	-			
11	16.2	1.50-1.40	m		
12	32.5	2.10, 1.45	m		
13	46.6	-			
14	158.5	-			
15	118.4	5.30	t		2.5
16	32.5	2.16	m		
17	53.8	2.21	dt		5.9, 8.8
18	19.7	1.01	s		
19	15.2	1.16	s		
20	39.3	2.75	ddd		12.1, 8.5, 5.9
21	170.4	-			
22	30.4	2.35	ddd	12.5	8.5, 6.0
		1.94	ddd	12.5	12.1, 10.8
23	78.1	4.18	ddd		10.8, 7.5, 6.0
24	64.2	2.81	d		7.5
25	57.3	-			
26	19.4	1.35	s		
27	24.8	1.37	s		
28	34.4	1.40	s		
29	23.6	1.51	s		
30	27.5	1.18	s		

OAc resonances at δ_H 2.10, 2.00 and δ_C 170.0, 169.7, 21.1, 20.8. Spectra run in $CDCl_3$ at 300 MHz for ^{1}H and 75.1 MHz for ^{13}C.

Initial inroads into the characterization of (1) were based on the ^{1}H-^{1}H COSY-45 spectrum (Figure 1). This revealed the sharp doublet for the epoxy oxymethine (δ 2.81, H-24) to be coupled to the δ 4.18 oxymethine (H-23) which in turn showed connectivity to methylene protons at δ 1.94 and 2.35 (H-22). In COSY-45 ^{2}J couplings are distinguishable from ^{3}J couplings by tilting of signals on the contour plot: this is clearly demonstrated for H-22 in Figure 1. The H-22 protons showed further coupling to another proton at δ 2.75 (H-20) and this was also coupled to a proton at δ 2.21 (H-17). The ^{1}H-^{1}H COSY showed H-17 to further couple to almost equivalent methylene protons (δ, 2.16, H-16) and the sequence was completed by a strong cross-peak for coupling between H-16 and the olefinic resonance at δ 5.30. The relationships gleaned from this analysis are summarized in Scheme 1.

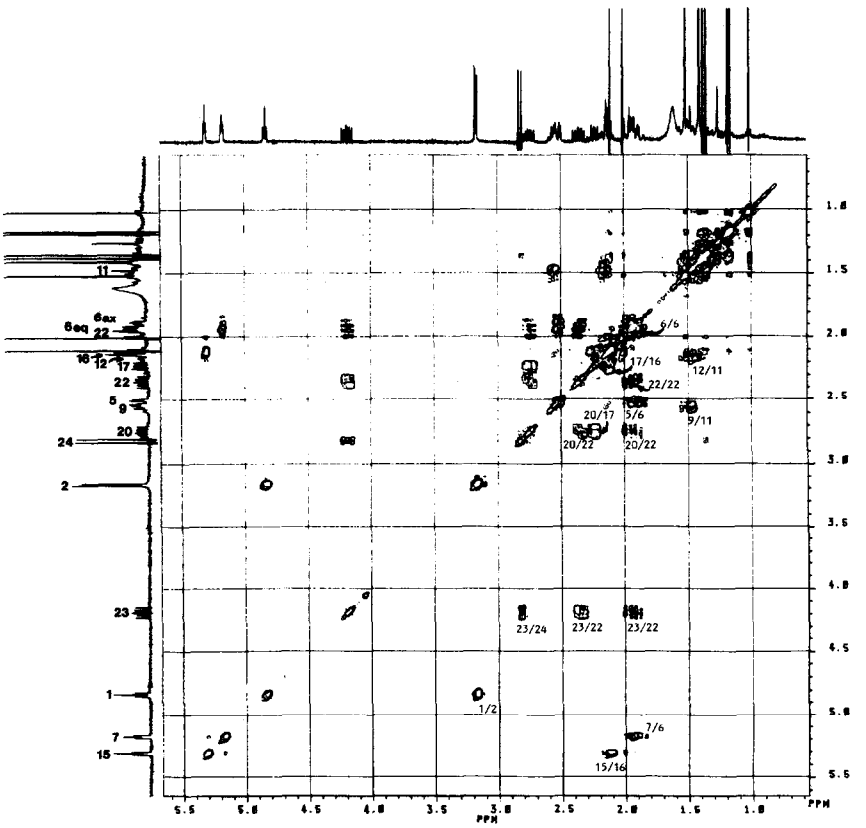
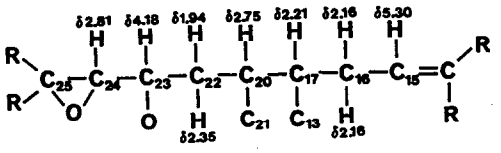


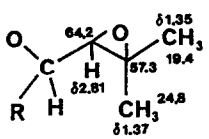
Figure 1. ^{1}H-^{1}H COSY-45 spectrum of phebaloparvilactone



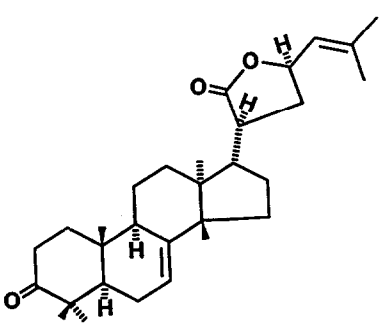
Scheme 1

The relationships identified in Scheme 1 were then further developed by reference to a Heteronuclear Multiple Bond Coherence (HMBC) study optimised to show ^{2}J and ^{3}J couplings, concentrating on the interactions of the ^{1}H methyl resonances (Figure 2) of (1). The oxymethine proton of the epoxide (δ 2.81) was found from the HMQC spectrum to have a direct connection to the carbon at δ_C 64.2. From the HMBC spectrum it is observed that this carbon shows ^{3}J interactions with two methyl singlets at δ 1.35 (δ_C 19.4) and δ 1.37 (δ_C 24.8, q) and that these methyl signals show ^{3}J correlation with one another and a ^{2}J interaction with a quaternary carbon at δ_C 57.3. This leads to the partial structure "a".

The HMQC spectrum revealed direct connectivity between the proton at δ 2.75 and the methine ^{13}C signal at δ_C 39.3. The proton resonance δ 2.75 is comparable with the H-20 proton (δ 2.74) of flindissone lactone (2), isolated from the oleoresin of *Aucoumea klaineana*.[7] Moreover, like flindissone lactone, the ^{1}H NMR spectrum of (1) was devoid of any H-21 resonance, indicating that there is a carbonyl group α to the proton at δ 2.75. The presence of two carbonyl carbons, other than acetoxyls, is inferred from the high intensity of the δ_C 170.4 resonance in the ^{13}C NMR spectrum, which suggests two equivalent signals.



"a"



(2)

The methine proton at δ 2.21 (H-17, Scheme 1) and the methylene protons at δ 2.16 (H-16) showed direct connectivity with carbons at δ_C 53.8 and 32.5, respectively. The carbon signal δ_C 53.8 was shown to have a ^3J correlation with the methyl singlet at δ 1.01; this must therefore be the C-13 methyl substituent. The protons of this methyl also showed ^3J correlations with carbons at δ_C 158.5 (C-14) and 32.5 (C-12) and a ^2J interaction with a quaternary carbon at δ_C 46.6 (C-13). The olefinic carbon must, obviously, link to the tertiary olefinic carbon at δ_C 118.4 and associated ^1H resonance at δ 5.30 thus completing the cyclopentane D-ring structure (e. g. "b").

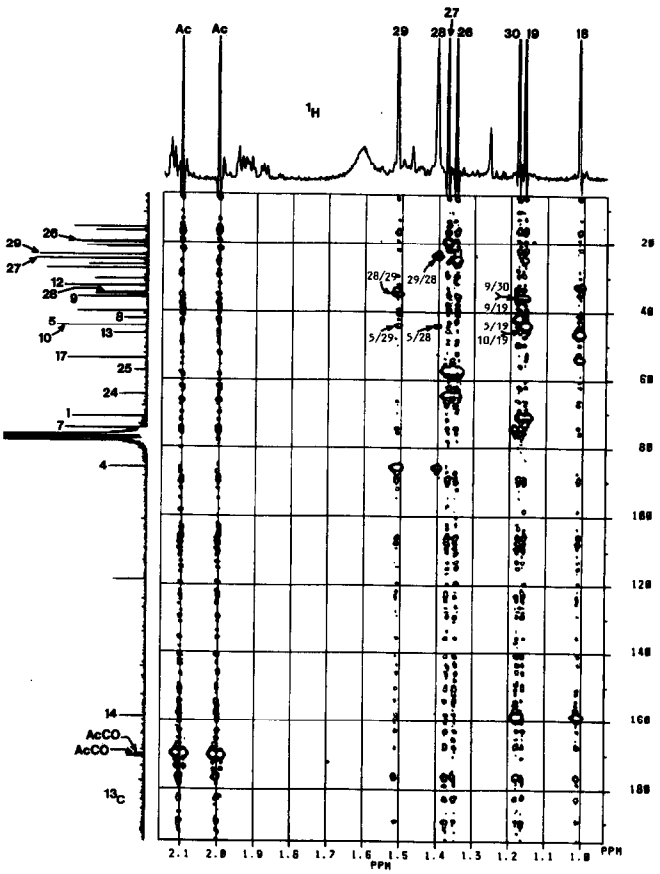
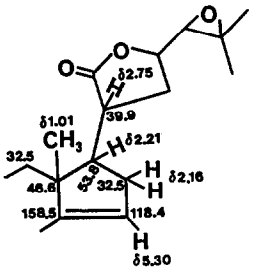


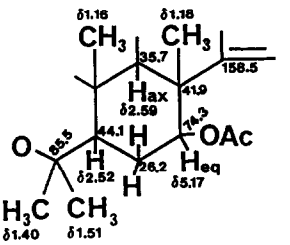
Figure 2. The ^1H methyl part of the HMBC spectrum of phebaloparvilactone (1)

Turning next to the quaternary olefinic carbon (δ_C 158.5, C-14) the HMBC spectrum revealed ^3J coupling to a further methyl singlet at δ 1.18; this must be the C-8 methyl substituent. Further ^3J correlations from this methyl link it to C-7 (δ_C 74.3, oxymethine) and to C-9 (δ_C 35.7, methine) and through a ^2J correlation to the C-8 resonance at δ_C 41.9. Having assigned the C-7 ^{13}C resonance direct connectivity to the oxymethine 1H triplet at δ 5.17 was indicated by the HMQC spectrum and the small ^1H J values for J_{7-6} (2.9 Hz each) established the equatorial nature of the proton. The HMQC spectrum also linked the C-9 ^{13}C resonance to a double doublet at δ 2.59 in the ^1H spectrum. Couplings of this proton (J = 11.0, 5.0 Hz) required that it be axial.

Returning to the ^1H-^1H COSY-45 spectrum (Figure 1) H-7 (δ 5.17) was seen to couple with a 2H multiplet between δ 2.00 and 1.85 (H-6, δ_C 26.3) which in turn interacted with a doublet of doublets at δ 2.52 (H-5, δ_C 44.1). In the HMBC spectrum (Figure 2) C-5 was found to exhibit ^3J correlations with three methyls at δ 1.16, 1.40 and 1.51. One of these methyls must be attributed to C-10 and the others to C-4. The methyls at δ 1.40 and 1.51 showed ^3J correlation with one another and with C-5 (δ_C 44.1) and a ^2J correlation with a resonance δ_C 85.5, which must be C-4 and has a chemical shift typical of an oxygen-bearing carbon. So, ring "A" is oxygenated at C-4 and must be either esterified or heterocyclic. These data established the fragment "c".



"b"



"c"

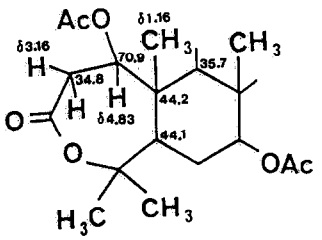
From the HMBC spectrum the carbon signals at δ_C 44.1 (C-5) and δ_C 35.7 (C-9) were observed to have correlations with the methyl at δ 1.16, which are the anticipated ^3J interactions of the C-10 methyl. Other connectivities seen for this methyl were a ^2J interaction with the C-10 carbon (δ_C 44.2) and a further correlation to an oxymethine resonance at δ_C 70.9 which can only represent the ^3J interaction to C-1. Through the HMQC spectrum the δ_C 70.9 resonance was linked with a triplet (1H) at δ 4.83, which suggests this to be the site for the second acetoxyl group.

Returning to the ^1H-^1H COSY-45 spectrum, the triplet at δ 4.83 showed an interaction with a 2H doublet at δ 3.16. This doublet revealed, through the HMQC spectrum showed direct connectivity with a carbon at δ_C 34.8 assignable to a CH_2 next to C=O (lactone). In the light of these data a further fragment "d" can be proposed.

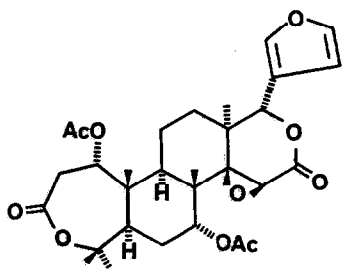
From the discussion so far spectral data are consistent with combination of the fragments "a" to "d" with the formation of two lactone systems to give structure (1) for phebaloparvilactone.

Having identified the gross structure of (1) the relative stereochemistry was then examined through a series of nuclear Overhauser enhancement difference experiments, based on the irradiation of the methyl resonances. The results of these are given in Figure 3.

The following important observations arise from the nOe experiments. Irradiation of the 18-Me enhanced H-9, H-20 and the acetoxyl groups, indicating that this methyl and these substituents are all on the same side of the molecule. The interaction with H-20 is of particular note as it requires the 18-Me and H-17 to be on opposite sides of the D-ring. Irradiation of the 19-Me caused the anticipated enhancement of the axial 29-Me signal (δ 1.51) and of H-1 and one of the H-2 protons, indicating that these substituents are on the same face of the molecule and, incidentally, as it enhanced H-1 the Me-19 is on the opposite face to Me-18, which enchanced the C-1 acetoxyl substituent. Irradiation of the two C-4 methyls had the anticipated effects. The axial methyl (Me-29), like Me-19, enhanced H-2, while the equatorial Me-28 caused enhancements of H-5 and H-6_{eq}. The Me-30 showed an nOe with H-6_{ax} and the H-7 oxymethine and must, therefore be on the opposite face to H-6_{eq} and H-5. The conclusions drawn about relative stereochemistry are shown pictorially in Figure 3. Only H-23 and H-24 relative stereochemistry remain ambiguous. It is presumed that, like all limonoids from the Rutaceae,[8] (1) is derived from tirucalla-7-ene and that, as in the closely allied compound 7α-nomilin acetate (3) the C-1 and C-7 acetoxy substituents are α (established in 3 by X-ray determination[9]). In 3 oxidations in rings A and B are comparable to 1, but in terms of side-chain modification and ring-D oxidation 1 lacks the full developments of the true limonoid.



"d"



(3)

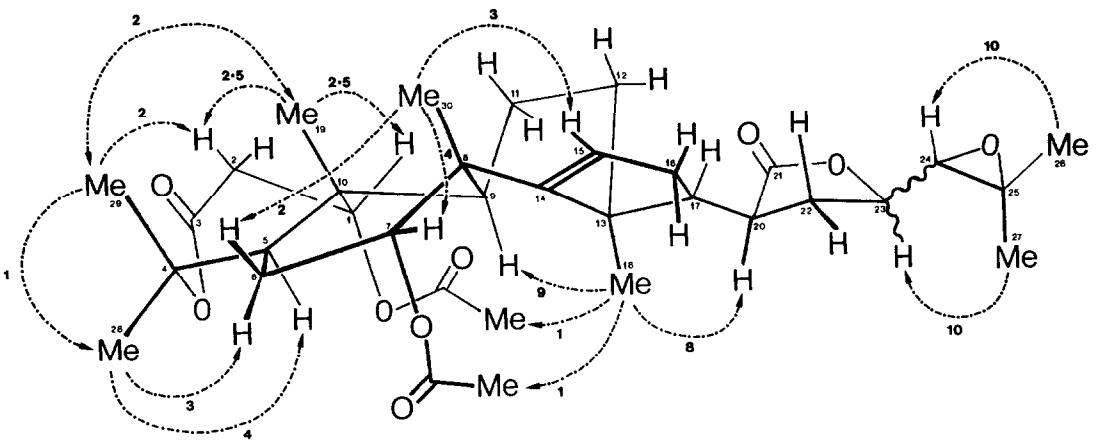


Figure 3

EXPERIMENTAL

Plant Material. Aerial parts were obtained from wild collected plants cultivated at the Australian National Botanic Gardens. Vouchers have been deposited under accession numbers ANBG 7708132, ANBG 7906577 and ANBG 8307889.

NMR Methods. The COSY-45 experiment was performed using the standard Bruker microprograms on a Bruker AC300 instrument. HMQC[10] was run with the BIRDD9 Bruker programme. ^{13}C Decoupling was performed during acquisition with a GARP sequence using a BFX5 amplifier. Recovery delays were optimised to null ^{12}C-H signals and found to equal 0.3 sec. HMBC[11] was run using the INVDR2LP programme. No ^{13}C decoupling was performed during acquisition; evolution delay was set at 70 ms, corresponding to 7Hz C-H coupling (^{2}J or ^{3}J). For homonuclear experiments matrixes were 256 x 1K data points and for the heteronuclear 256 x 2K data points. Sine bell multiplication was applied in both dimensions before Fourier transformation, except for HMBC and HMQC experiments where a shift of 60° was applied. NOe experiments were run on a Bruker WH360 instrument using the standard NOEDIFF microprogram. Methyl singlets were irradiated at 41 dB below 0.2 watt during the preacquisition delay of 10 s, with a 2 s acquisition time. A 1 Hz line broadening function was applied to the FID before Fourier transformation and subtraction of the control spectrum (irradiation at 0.1 ppm) from each "on resonance" irradiation spectrum.

Extraction of phebaloparvilactone. Preliminary TLC examinaton of the petrol (b.p. 60-80°C), EtOAc and MeOH extracts of the collection nos. 8307889, 7906577c and 7708132 revealed that they were identical. Powdered leaves of bulked collections (122 g) were

extracted in a Soxhlet successively with petroleum ether (60-80°C), EtOAc and MeOH. TLC on silica gel showed, under UV light, that both the petrol and EtOAc extracts contained similar components with R_f values 0.67, 0.56, 0.38 and 0.20 (EtOAc:petrol; 4:1) and these extracts were bulked. TLC examination of the MeOH extract did not reveal the presence of any major compounds. The combined petrol and EtOAc extracts were concentrated by a rotary film evaporator keeping the water bath at a temperature below 40°C and then subjected to VLC over TLC grade silica gel. Elution of the column with petrol (60-80°C) followed by mixtures of petrol and increasing amounts of EtOAc and finally EtOAc gave the compounds successively. The compounds with R_f values 0.67 and 0.56 were found to be hydrocarbons on routine analysis of 90 MHz ^1H NMR spectra and were not studied further. However, the compounds with R_f values at 0.38 and 0.20 were found to be mixtures from the study of their 90 MHz ^1H NMR spectra. The R_f 0.20 band was subjected to PTLC (silica gel, $CHCl_3$: Me_2CO; 3:1) to yield phebaloparvilactone (5 mg) and scopoletin (2 mg).

Properties of phebaloparvilactone. White amorphous solid. $[\alpha]_D$ -31° (c 0.2, $CHCl_3$); FAB-MS : m/z 601 $[M+H]^+$ and other peaks at m/z 481, 409 and 71; FT-IR υ max(KBr): 2878, 2928, 1775, 1731, 1375, 1234, 1167, 1120, 1025, 929, 816 cm^{-1}. ^1H NMR and ^{13}C NMR - see Table 1.

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