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Phytochemistry 67 (2006) 1708–1715
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Structures, biogenetic relationships, and cytotoxicity of pimarane-derived diterpenes from *Petalostigma pubescens*

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Received 17 August 2005; accepted 21 September 2005
Available online 18 November 2005

This article is dedicated to Professor Rodney Croteau on the occasion of his 60th birthday.

Abstract

Extraction of *Petalostigma pubescens* heartwood followed by chromatographic purifications and crystallizations afforded five tricyclic diterpenes: 5,9-syn-rosanes petalostigmones A and B (**1** and **2**), the erythroxylane petalostigmone C (**3**), the norditerpene lactone pubescenone (**4**), and the known *ent*-cleistanthane diterpene (−)-sonderianol (**5**). The structures and relative stereochemistry were elucidated by means of spectroscopic methods, chemical correlations, and, in the cases of **1** and **4**, by X-ray crystallographic analyses. The new isolates **1**–**4** are assumed to belong to the same absolute configurational family (9αCH_3) of *ent*-pimarane-derived diterpenes as the known co-occurring (−)-**5** (10αCH_3). Biogenetic schemes originating from a common *ent*-copalyl diphosphate intermediate are presented to rationalize the structures of these natural products. A novel ring contraction–ring expansion mechanism is suggested to account for the 7-membered B ring of pubescenone. Compounds **1**–**5** were evaluated for their cytotoxicity; sonderianol (**5**) showed the highest activity against mouse leukemia cell lines L1210, P388 and mouse liver cancer cells HEPA1c1c7.

Keywords: *Petalostigma pubescens*; Euphorbiaceae; Heartwood; Diterpene; Rosane; Erythroxylane; Nor diterpene; Cleistanthane; Biogenetic pathways

1. Introduction

Species of the family Euphorbiaceae are known as a source of several structural classes of diterpenes, many of which exhibit antitumor, cytotoxicity, tumor-promoting, or antimicrobial activity (Evans and Taylor, 1983; Adolf and Hecker, 1977). *Petalostigma* is an Australian genus of the Euphorbiaceae which has not been investigated chemically. We now report the isolation and structure elucidations of 5,9-syn rosadienones (**1**) and (**2**), the *ent*-erythroxydienone (**3**), *ent*-norditerpene lactone (**4**), and the known *ent*-cleistanthane diterpene (−)-**5** from the heartwood of *Petalostigma pubescens* (Fig. 1).[1]

2. Results and discussion

2.1. Isolation and structure elucidations

Toluene and chloroform extracts prepared from the powdered heartwood of *Petalostigma pubescens* were frac-

[1] The systematic names for the *ent*-rosane and *ent*-erythroxylane diterpenes are as follows: *ent*-12α-hydroxy-5βH-rosa-1(10),15-dien-2-one (**1**), *ent*-5βH-rosa-1(10),15-dien-2,12-dione (**2**), and *ent*-12α-hydroxyerythroxy-1(10),3,15-trien-2-one (**3**) (Rigaudy and Klesney, 1979; Connolly and Hill, 1991). A systematic name for pubescenone (**4**) was derived from that given to fischeria A (Pei et al., 1999) by Chemical Abstracts: (6-hydroxy-2,4a,7,7-tetramethyl-3-oxo-2-vinyl-decahydrobenzocycloheptен-5-ylidene)acetic acid lactone.

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doi:10.1016/j.phytochem.2005.09.026

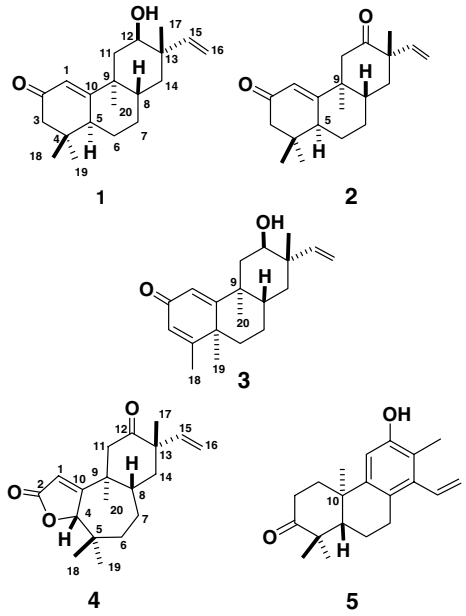


Fig. 1. Structures of the diterpene ketones (**1**–**5**) isolated from *Petalostigma pubescens*.

tionated by silica gel column chromatography to yield four new diterpenoids: petalostigmones A, B, and C, and pubescenone (**1**–**4**), and in addition the known cleistanthane, sonderianol (**5**) (Fig. 1).

Petalostigmone A (**1**), obtained as a white crystalline solid (m.p. 196–197 °C), was shown to have the molecular formula $C_{20}H_{30}O_2$ according to the HRESIMS molecular ion peak observed at *m*/*z* 303.2329 [M + 1], and from analysis of its ^{1}H and ^{13}C NMR spectroscopic data. The IR spectrum of **1** displayed strong absorptions at 1659 and 3430 cm^{-1} indicating the presence of an α,β-unsaturated ketone and a hydroxyl group. The ^{1}H NMR spectra (Table 1) exhibited three singlets that integrated for four methyl groups (δ 1.02, 0.93 and 1.11 ppm), and the characteristic pattern for a vinyl group [δ 5.84 *dd* (*J* = 17.6, 10.9 Hz), 5.12 *dd* (*J* = 17.6, 0.9 Hz), 5.10 *dd* (*J* = 10.9, 1.0 Hz)], attached to a quaternary carbon which is usually present in pimaranes and related diterpenes. The signal for the vinyl hydrogen of the α,β-enone moiety appears as a singlet at δ 5.87, and no correlations to this signal were observed in the COSY spectrum. The appearance of nine signals for both methyls and methines, and eleven signals for both methylene and quaternary carbons in the ^{13}C NMR (Table 2) and APT spectra add further support for a pimarane diterpene skeleton.

Acetylation of **1** (Ac_2O, pyr, 25 °C) afforded a crystalline acetate derivative, confirming the presence of an unhindered hydroxyl group. The location of the hydroxyl group (12β OH) and the complete assignments of the ^{1}H and ^{13}C NMR resonances were deduced from HMQC and HMBC experiments, together with comparisons of the NMR data with those for some related diterpenes found in the literature (Geis and Becker, 2000; Mdee et al., 1998). The structure and relative stereochemistry were confirmed by an X-ray crystallographic analysis (Fig. 2).

Reduction of the enone in **1** (*i*Bu_2AlH, toluene, −78 °C) (Wilson et al., 1970) provided a 38:62 mixture of chromatographically separable diols **6** and **7** (Scheme 1).

Table 1
^{1}H NMR spectroscopic data δ_H *m* (*J* in Hz) for diterpenes **1**–**4** ($CDCl_3$, 500 MHz)

H	1	2	3	4
1	5.87 *s*	5.72 *s*	6.20 *s*	5.94, *d* (1.7)
2	–	–	–	–
3α	2.11 *d* (15.5)	2.28 *d* (16.1)		–
3β	2.26 *d* (15.5)	2.12 *d* (15.6)	6.05 *s*	–
4	–	–	–	5.01, *d* (1.7)
5	2.34 *dd* (12.1, 4.5)	2.23 *dd* (12.7, 3.8)	–	–
6α	2.01–2.04 *m*	2.09–2.13 *m*	1.40 *m*	1.39, *dt* (15.4, 4.0)
6β	1.35 *qd* (12.2, 4.5)	1.48 *qd* (12.8, 3.8)	1.20 *m*	1.57–1.73 *m*
7α	1.49–1.55 *m*	1.43 *qd* (12.8, 3.8)	1.50 *m*	1.57–1.73 *m*
7β	1.40 *qd* (12.2, 3.2)	1.70–1.74 *m*	1.65 *m*	1.57–1.73 *m*
8	1.47 *m*	1.99 *tt* (12.2, 3.5)	1.49 *m*	2.20 *ddd* (13.3, 9.0, 3.5)
9	–	–	–	–
10	–	–	–	–
11α	1.76, app *dd* (12.1, 4.0)	2.31 *d* (13.5)	2.03 *dd* (12.5, 4.6)	2.25, *d* (14.1)
11β	1.63, app *t* (11.8)	2.76 *d* (13.5)	1.51 *t* (12.0)	2.80, *dd* (14.2, 0.9)
12	3.80, app *dd* (11.6, 3.4)	–	3.83 *dd* (11.6, 4.5)	–
13	–	–	–	–
14α	1.22 *dd* (13.3, 2.5)	1.80 *t* (13.5)	1.49 *m*	1.92 *t* (13.7)
14β	1.46 *t* (13.0)	1.59 *dd* (14.0, 3.6)	1.50 *m*	1.61 *dd* (14.0, 3.9)
15	5.84 *dd* (17.6, 10.9)	6.20 *dd* (17.6, 10.9)	5.86 *dd* (17.3, 10.7)	6.16, *dd* (17.8, 11.2)
16E	5.10 *dd* (10.9, 1.0)	5.15 *dd* (10.9, 0.9)	5.11 *dd* (10.7, 1.0)	5.18 *dd* (11.0, 0.7)
16Z	5.12 *dd* (17.6, 0.9)	5.06 *dd* (17.8, 0.8)	5.12 *dd* (17.3, 1.0)	5.08 *dd* (17.8, 0.7)
17	1.02 *s*	1.31 *s*	0.98 *s*	1.36 *s*
18	0.93 *s*	0.99 *s*	1.98 *s*	1.24 *s*
19	1.02 *s*	1.00 *s*	1.37 *s*	0.70 *s*
20	1.11 *s*	1.01 *s*	1.23 *s*	1.21 *d* (0.9)

Table 2
^{13}C NMR spectroscopic data (δ in ppm) for diterpenes **1**–**4** (125 MHz, $CDCl_3$)

Carbon	1	2	3	4
1	119.8	119.6	123.3	118.4
2	200.8	200.3	187.0	179.8
3	49.6	48.6	125.9	–
4	34.6	34.4	167.3	89.7
5	44.5	44.9	43.4	43.2
6	28.6	28.8	39.3	42.9
7	28.7	28.2	24.5	34.4
8	41.3	42.1	39.9	40.0
9	42.8	44.9	41.7	39.2
10	172.1	170.6	173.6	172.6
11	39.7	48.6	41.3	53.8
12	72.0	213.1	71.9	210.6
13	42.3	50.6	42.3	50.2
14	39.4	40.3	35.8	42.6
15	147.5	142.3	147.3	141.6
16	113.3	113.0	112.9	113.5
17	15.0	17.8	14.9	18.8
18	25.3	26.2	19.2	20.1
19	27.8	28.5	27.6	30.1
20	18.4	23.6	19.4	23.5

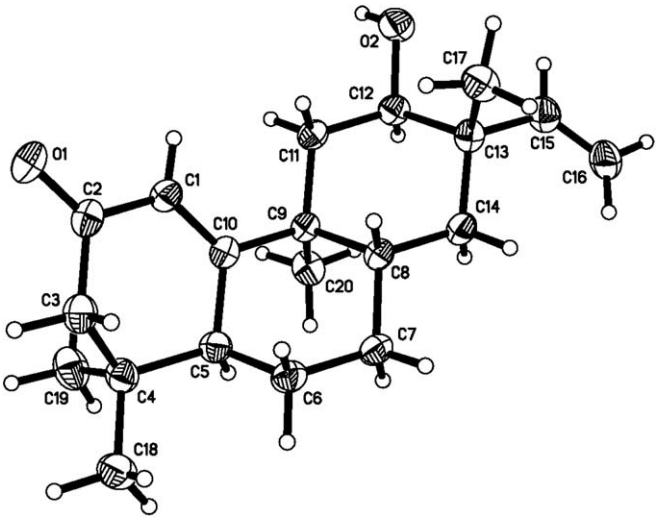
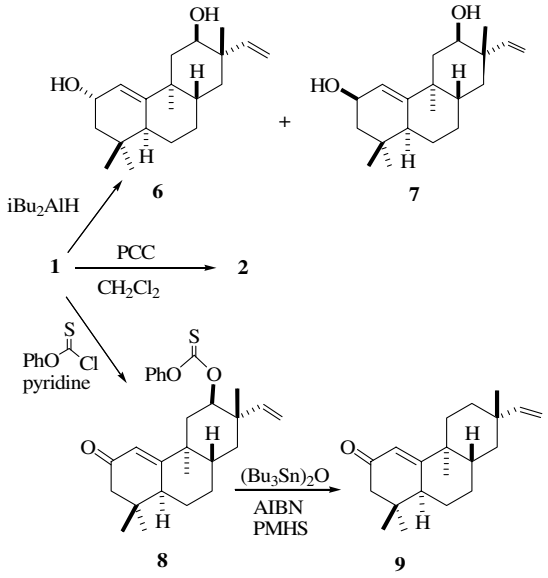


Fig. 2. The ORTEP drawing of **1**.

The major and more polar isomer was obtained in crystalline form and tentatively assigned as the 2β,12β-diol (**7**). The C-12 hydroxyl group of **1** was removed by tin hydride reduction (Tormo and Fu, 2002) of the phenyl thionocarbonate derivative **8** to give dienone **9**, albeit in only 11% yield. Isomeric diols **6** and **7**, and the parent dienone **9**, are plausible biosynthetic precursors to petalostigmones A and B.

Petalostigmone B (**2**) was isolated as a white crystalline solid (m.p. 110–114 °C). The HREIMS showed *m/z* 300.2089 corresponding to $C_{20}H_{28}O_2$. Its IR spectrum displayed two carbonyl peaks at 1656 and 1615 cm^{-1} and no absorption in the hydroxyl region. The ^1H NMR spectrum was very similar to that of **1**, except for the lack of signals around δ 3–5 ppm indicating the absence of the methine



Scheme 1. Reactions of petalostigmone A.

Table 3
NOE enhancements in the 500 MHz ^1H NMR spectrum of petalostigmone B (**2**) in $CDCl_3$ after irradiation at 1.99, 2.76, and 5.72 ppm

Irradiation at 1.99 ppm (H8β)		Irradiation at 2.76 ppm (H11β)		Irradiation at 5.72 ppm (H1)	
Shift (ppm)	NOE (%)	Shift (ppm)	NOE (%)	Shift (ppm)	NOE (%)
1.31 (H17)	5.3	1.31 (H17)	5.0	2.31(H11α)	7.0
1.48 (H6β)	2.9	1.99 (H8β)	6.2	2.76(H11β)	7.6
1.59 (H14β)	4.4	2.31 (H11α)	29.1		
1.72 (H7β)	2.3	5.72 (H1)	15.7		
2.76 (H11β)	3.8				

proton at C-12. Also the ^{13}C NMR spectrum indicated the absence of signals for *C*HOH, instead showing two downfield carbonyl carbons at δ 200.3 and 213.1 ppm. The stereochemical assignments at C-3, C-6, C-7, C-8 and C-11 were made with the aid of NOE experiments (Table 3). Upon irradiation of the C-8 proton, H-6β, H-7β, H-11β, H-14β and H-17 showed NOE enhancements. Additionally, H-l, H-8β, and H-17 exhibited positive NOE responses upon irradiation of H-11β. The structure of petalostigmone B (**2**) was confirmed by oxidation of **1** with pyridinium chlorochromate in dichoromethane (Herscovici and Antonakis, 1980) which gave the corresponding 2,12-dione (80%) that was identical to **2** by mp, mixed mp, and NMR spectral comparisons.

Petalostigmone C (**3**) was obtained as colorless needles (m.p. 180–184 °C). The molecular formula was $C_{20}H_{28}O_2$ based on the HREIMS (*m/z* 300.2084) and the 20 signals observed in the ^{13}C NMR spectrum (Table 2). The IR spectrum showed absorptions at 1656 and 3418 cm^{-1} similar to those in the spectrum of petalostigmone A, indicating that the oxygens are again present as α,β-enone and hydroxyl functionalities. The ^1H NMR spectrum displayed two downfield singlets at δ 6.20 and 6.05 ppm, suggesting the

Table 4
NOE enhancements in the 500 MHz ^{1}H NMR spectrum of petalostigmone C (**3**) in $CDCl_3$ after irradiation at 1.00, 1.23, 3.83, and 6.20 ppm

Irradiation at 1.00 ppm (H17)		Irradiation at 1.23 ppm (H20)		Irradiation at 3.83 ppm (H12α)		Irradiation at 6.20 ppm (H1)	
Shift (ppm)	NOE (%)	Shift (ppm)	NOE (%)	Shift (ppm)	NOE (%)	Shift (ppm)	NOE (%)
1.49 (H8)		1.37 (H19)	1.4	1.23 (H20)	10.5	1.51 (H11β)	4.7
1.50 (H14β)		1.49 (H14α)	3.2	1.49 (H14α)	2.7	2.03 (H11α)	10.0
1.51 (H11β)		1.50 (H7α)	2.9	2.03 (H11α)	4.6		
5.12 (H16Z)	2.7	2.03 (H11α)	0.6	5.12 (H16Z)	0.6		
5.86 (H15)	0.8	3.83 (H12α)	3.7	5.86 (H15)	8.1		

presence of two isolated vinyl protons attached to positions 1 and 3 of a dienone moiety. The spectrum also displayed three quaternary methyls, a vinyl methyl, an oxygenated methine proton, and a vinyl group. The ^{13}C NMR spectrum showed the presence of six C=C carbons at δ_c 123.3, 125.9, 167.3, 173.6, 147.3, 112.9 ppm for C-1, C-3, C-4, C-10, C-15 and C-16, respectively, and in addition the ketone carbonyl carbon at 187.0 ppm. The assignments for H-6, H-7 and H-14 were established by COSY and HMQC correlations.

The spatial relationships within the molecule were deduced from NOE experiments (Table 4). In particular, irradiation of the C*H*OH proton at C-l2, produced NOE enhancements at H-11α, H-14α, H-15 and H-20, indicating that these protons are all on the same face of the molecule (α face). Positive NOE responses were observed at H-8, H-14β, and H-11β, upon irradiation of the C-17 methyl proton signal, proving those protons are on the other side of the molecule (β face). The stereochemistry at C-5 was determined to be 5αCH_3 based on the positive NOE response (1.4%) of the C-19 methyl protons upon irradiation of C-20 methyl proton signal. From these structural elements, we conclude that petalostigmone C has structure **3** and is a diterpenoid belonging to the erythroxylane (13-epi-dolabrane) group (Kijjoa et al., 1995, 1994; Nagashima et al., 1991; Nagahama and Tajima, 1996; Abdel-Kader et al., 1993).

Pubescenone (**4**) was obtained in crystalline form as needles (m.p. 145–148 °C). The molecular formula was assigned as $C_{19}H_{26}O_3$ from its HREIMS (*m/z* 302.1886). The IR spectrum shows two peaks at 1754 and 1705 cm^{-1} for two carbonyl groups, the former for an α,β-unsaturated γ-lactone, and no absorption was observed in the hydroxyl region. The ^{13}C NMR and APT spectra (Table 2) reveal the presence of four unsaturated carbons at δ_c 118.4, 172.6, 141.6, 113.5 ppm for C-1, C-10, C-15 and C-16, respectively, two carbonyl carbons at δ_c 210.6 and 179.8 ppm and in addition one oxygenated methine carbon at δ_c 89.7 ppm.[2] The ^{1}H NMR spectrum (Table 1) displayed the characteristic peaks for a vinyl group (δ 6.16, 5.18 and 5.08 ppm), one olefinic methine (5.94 ppm), an oxygenated methine proton (5.01 ppm) and four quaternary methyl groups (0.70, 1.21, 1.24, 1.36 ppm). The overall structure and relative configuration of pubescenone were confirmed by X-ray analysis (Fig. 3), and by comparisons with the spectroscopic data for the related 12-deoxy derivative fischeria A previously isolated from *Euphorbia fischeriana* (Pei et al., 1999).

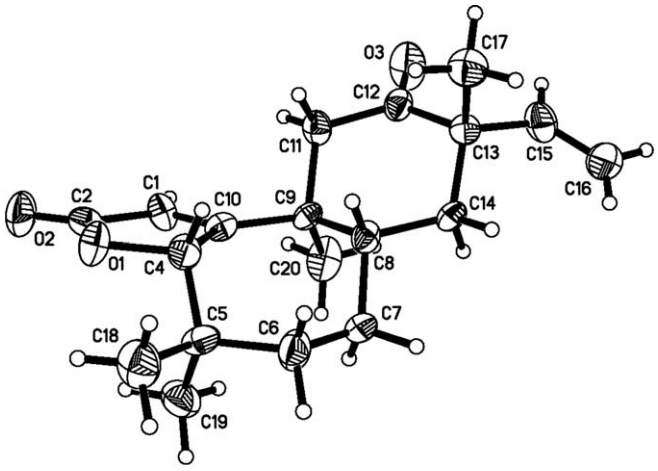
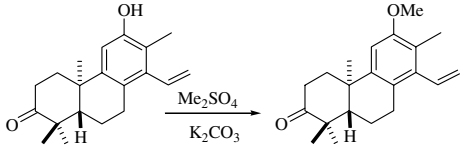


Fig. 3. The ORTEP drawing of **4**.

Sonderianol (**5**) was obtained as a pure colorless oil with an optical rotation of $[\alpha]_D^{25}$ −9.4°. The NMR and mass spectroscopic data were in agreement with those of the known *ent*-cleistanthane diterpene, (−)-sonderianol isolated from *Croton sonderianus* (Carveiro and Silveira, 1982). However, since the optical rotation and the physical form of the isolated compound did not agree with those reported previously for (−)-sonderianol (Carveiro and Silveira, 1982), we prepared the known crystalline *O*-methyl derivative (−)-**10** (Tan et al., 2002) (Scheme 2).

The m.p. (97–99 °C), optical rotation ($[\alpha]_D^{25}$ −63.5°), and proton NMR spectroscopic data for **10** are in good agreement with the reported data for the methyl ether



(-)-5 (-)-10

Scheme 2. Methylation of (−)-sonderianol.

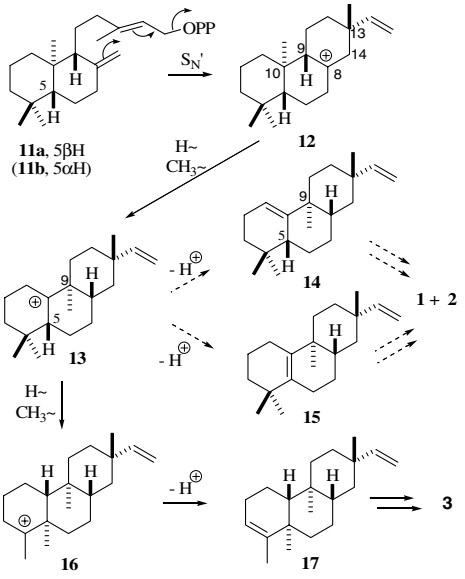
[2] The positional numbering adopted for **4** follows the same pattern as those of **1**–**3**, for ease of data comparisons. This numbering scheme differs from that in the systematic name[1] for **4**.

derivative of (−)-sonderianol (m.p. 106–107 °C, $[\alpha]_D^{25}$ −68°) (Denton et al., 2001; Gunasekera et al., 1979). This derivatization serves not only to establish the identity of sonderianol isolated in this work but also proves that the compound is an *ent*-diterpene as shown in structure **5**. In the absence of any further evidence, we have provisionally assigned the same absolute configuration to all five co-occurring diterpenes on the assumption that all are biosynthesized from *ent*-copalyl diphosphate (**11a**).

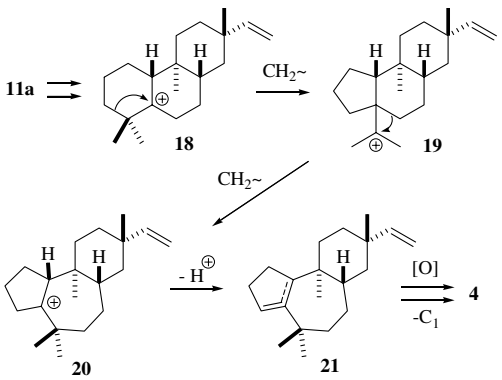
2.2. Biogenetic relationships of petalostigma pubescens diterpenes

The similar tricyclic hydrophenanthrene nucleus of diterpenes **1**–**3** and **5**, and especially the highly characteristic geminal, or in the case of the *ent*-cleistanthane (−)-**5** vicinal, methyl and vinyl substituents on ring C of all five compounds strongly suggest their biogenetic origin from a common *ent*-pimaren-8-yl$^+$ ion intermediate **12** generated by S_N' cyclization of *ent*-copalyl diphosphate (CPP, **11a**) as shown in Schemes 3 and 4 (Dev and Mitra, 1985; MacMillan and Beale, 1999). Hydroxy dienone **1** and its diketone congener **2** are clearly *ent*-rosane diterpenes, likely biosynthesized by oxidative transformations of an *ent*-rosa-5,15-diene or *ent*-rosa-1(10),15-diene (**14** or **15**) precursor that is formed by successive 9 → 8 hydride and 10 → 9 methyl shifts to rosen-10-yl$^+$ ion **13**, followed by proton elimination (Scheme 3).

What is unusual, and unprecedented to our knowledge, is the syn relationship of 5α H and 9α CH_3 in rosane derivatives **1** and **2**. If the 5α hydrogen was actually derived intact from its bicyclic progenitor, the *ent*-CPP precursor must have had a *cis* AB ring fusion, i.e. (**11b**). Although a step-wise cyclization of (*E*,*E*,*E*)-geranylgeranyl PP to an AB cis *ent*-CPP (**11b**) seems to be mechanistically feasible, none of the numerous known natural labdane diterpenes (Dev and Mitra, 1985; Connolly and Hill, 1991; Hanson, 2004) have been proven to have that anomalous stereochemistry. More likely alternatives are illustrated in Scheme 3, i.e., elimination of H5β, either in an initial cyclization to **15** or in the following oxidative processes, and later reincorporation of a proton at C-5 with the opposite 5α configuration during the conversion of the *ent*-rosadiene intermediate to **1** and **2**.



Scheme 3. Biogenesis of petalostigmones A, B, and C (**1**–**3**).



Scheme 4. Proposed biogenesis of pubescenone by a ring expansion–ring contraction mechanism and oxidative modifications of ring A.

ent-Rosa-1(10), 15-diene (**14**) with 5,9-anti stereochemistry probably exists in a rather strained half-chair/boat/chair conformation of the A–B–C fused rings. The corresponding anti epimers of **1** and **2** with the 5βH configuration would also adopt similar strained conformations. This raises the possibility that these unstable isomers were originally present in the heartwood, and that they underwent enone/dienol isomerization and epimerization at C5 to petalostigmones **1** and **2** during the extractions and purifications. However, a very similar diterpene, 3β-hydroxy-rosa-1(10), 15-dien-2-one (hugorosenone), having 5αH/9βCH_3 anti stereochemistry was isolated by apparently similar extractions and chromatographies (Mdee et al., 1998). Hence, the origin of the anomalous 5,9-syn stereochemistry of these *ent*-rosane diterpenes is uncertain at this time.

Dienone **3**, an erythroxylane diterpene,[1] is probably biosynthesized from *ent*-13-epi-dolabradiene (**17**), (Nagahama and Tajima, 1996; Ohgaku et al., 1984), or its endocyclic $\Delta^{3,15}$ isomer, by oxidations on the A and C rings. The aromatic ring C of the *ent*-cleistanthane diterpene (−)-**5** is presumably formed by oxidation and aromatization of an *ent*-cassadiene intermediate (Yajima et al., 2004; Cho et al., 2004) formed by 14 → 8 hydride shift and 13 → 14 vinyl rearrangement of the same *ent*-pimaren-8-yl$^+$ ion **12**.

The presence of the 5,5,9-trimethylcycloheptane B ring in keto butenolide **4** obviously signals the occurrence of an unusual rearrangement and the loss of one carbon during the biosynthesis of this novel tricyclic ring system. We propose that the *ent*-rosa-15-en-5-yl$^+$ ion precursor (**18**) to *ent*-epi-dolabradiene undergoes first ring contraction of the A ring to hydrindanylcarbinyl$^+$ cation (**19**) and then

expansion of the B ring to the hydroazulenyl^{+} ion (**20**) after which proton elimination would give one of the isomeric dienes (**21**), or alternatively the corresponding tertiary alcohol by water capture (Scheme 4).

The 5–7–6 ring system together with the distinctive pattern of gem dimethyl, angular methyl, and geminal methyl and vinyl substituents on **21** would be a new structural type for the diterpene family of natural products. Although numerous diterpenes having either the same 5–7–6 nucleus (dolastanes, sphaeroanes, rahmnopholanes, and daphnanes) or a tetracyclic ring system with an imbedded 5–6–7 partial structure (grayanotoxanes and tiglianes) are known (Connolly and Hill, 1991), none have the geminal methyl and vinyl groups characteristic of pimarane-derived structures. One exception is parryin, the 6–7–5 structure of which is presumably derived by a pinacolic-type rearrangement of the B and C rings of a pimarene precursor (Touche et al., 1997). Precedent for the proposed ring contraction **18** → **19** is available in the biogenesis of the fungal metabolite pleuromutilin (Arigoni, 1968; Hasler, 1979). The two energy barriers in the rearrangement sequence **18** → **19** → **20** should be readily surmounted since all three carbocation intermediates are tertiary. Oxidative modifications of the A ring of **21** could plausibly lead to loss of one ring carbon and formation of the butenolide residue in nor diterpene **4**.

Although formation of the 7-membered ring in **4** by the rearrangements shown in Scheme 4 affords an attractive rationale for the novel structure of this diterpene, the alternative possibility of an unusual skeletal mutation occurring during oxidative metabolism of an *ent*-rosadiene precursor should be kept in mind. For example, oxidative ring contraction of 7β-hydroxykaurenoic acid leads to the 5-membered B ring on the biosynthetic pathway to the gibberellins, and a D-ring bridge rearrangement of gibberellin GA_9 to antheridic acid has been postulated (MacMillan and Beale, 1999; MacMillan, 1997; Mander, 2003). Furthermore, numerous diterpenes are known which have oxidatively modified structures not readily derivable by precedented biogenetic cyclizations and rearrangements (Dev and Mitra, 1985; Connolly and Hill, 1991; Hanson, 2004).

The four isolated compounds showed mild cytotoxic activity against both mouse leukemia cell lines (L1210 & P388). (−)-Sonderianol displayed 100% growth inhibition at a concentration of 50 μg/mL and also exhibited moderate toxicity against mouse liver cancer cells HEPAlclc7 with IC_{50} 0.403 mM.

3. Experimental

3.1. Plant material

Petalostigma pubescens heartwood was kindly supplied and identified by Dr. Eugene Dimitriadis, Xyloaustralis, 44 Hamono RD., Neerim, Vic. 3831, Australia, in 2003. A vouchered plant sample from a second collection was submitted to the Brisbane Herbarium, AQ 751251 (collector number PB-1). Extraction of the milled heartwood of the voucher sample and fractionation by chromatography were carried out as described in detail below. The presence and identity of the same five diterpenes **1**–**5** were established by crystallization of the major components (**1** and **3**), and by comparisons of TLC behavior and ^{1}H NMR spectra with those of the previously isolated compounds.

3.2. Extraction and isolation

The dried milled heartwood (475 g) was extracted at room temp. by soaking over night, first with toluene (3 × 2 L), and then with chloroform (2 × 2 L). Filtration and evaporation of the solvents using a rotary evaporator (40 °C) gave yellow oily residues (9.2 and 9.6 g, respectively). The toluene extract (8.5 g) was loaded onto a silica gel column (50 × 3.5 cm), eluting first with hexane and then with increasing amounts of EtOAc (2–50%) in hexane, while collecting 25 × 200-mL fractions. Similar fractions according to TLC analyses were combined, immediately evaporated to dryness, and kept in the freezer for further fractionation.

Petalostigmone A (**1**) (450 mg) was directly crystallized from fractions 16–20 (elution with 25% EtOAc in hexane) by concentrating and allowing the solution to stand overnight at room temp. Petalostigmone C (**3**) (30 mg) was similarly obtained from fractions 23–25 (elution with 30% EtOAc in hexane). Fractions 10 and 11 (3.5 g) were further purified by repeated chromatography on silica gel column using hexane with increasing proportions of EtOAc as eluents. Sub-fractions eluted with 10% EtOAc in hexane were repeatedly column chromatographed to obtain the major component, petalostigmone B (**2**) (196 mg), in pure crystalline form and somewhat impure sonderianol (**5**, est. 45.8 mg, 85% purity by NMR analysis).

Further purification by repeated preparative TLC provided pure sonderianol (**5**) for spectral analyses and optical rotation measurement. The rest of the fraction was used for preparation of the methyl ether derivative (**10**). Pubescenone (**4**, 12.8 mg) was isolated by repeated column chromatography of the fraction eluted with 14% EtOAc in hexane. The chloroform extract (8.9 g) was fractionated by column chromatography in the same way as the toluene extract to give petalostigmone A (**1**) (550 mg) and petalostigmone C (**3**) (185 mg).

3.3. Petalostigmone A (*1*)

White crystals; m.p. 196–197 °C; $[\alpha]_D^{25}$ − 86.2° (c 1.0, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε) 238 (1.4) nm; IR ν_{max} ($CHCl_3$) 3430, 2925, 2870, 1659, 1216 cm^{-1}; ^{1}H and ^{13}C NMR: see Tables 1 and 2; HRESIMS *m*/*z* 303.2329 (calcd. for $C_{20}H_{31}O_2$ 303.2324) [M + 1]; EIMS *m*/*z* (rel. int.): 302 (68) [M]$^{+}$, 287 (19), 258 (14), 246 (20), 216 (55), 194 (37), 152 (32), 133 (37), 121 (36), 107 (100), 91 (67), 79 (59);

Crystal data: Monoclinic crystals, $C_{20}H_{30}O_2$, mol. wt = 302.44, crystal size $0.50 \times 0.44 \times 0.26$ mm^3, space group P21.

3.4. Acetate derivative of petalostigmone A

Acetylation of compound **1** (6.6 mg, 0.7 mmol) with Ac_2O/pyridine (1:1, 0.5 mL; 20 h, room temp.), precipitation with ice cold-water and filtration gave 6.0 mg (80%) of the acetate derivative as a crystalline solid: m.p. 126–128 °C; $[\alpha]_D^{25}$ −121° (*c* 0.4, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.80 (*s*, H-1), 5.76 (*dd*, *J* = 18, 11 Hz, H-15), 4.98 (*dd*, *J* = 18, 1.1 Hz, H-16Z), 4.96 (*dd*, *J* = 8, 1.4 Hz, H-16E), 2.01 (*s*, CH_3CO_2), 1.16, 1.07, 1.01, 0.95 (4 s, 4CH_3); HRESIMS *m*/*z* 345.2492 for $C_{22}H_{33}O_3$ (calcd. 345.2430) [M + 1].

3.5. Characterization data for diols 6 and 7 from petalostigmone A

See Supporting Material for the experimental procedure. Data for minor diol **6**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.82 (*dd*, *J* = 17.4, 10.6 Hz, H-15), 5.44 (bs, H-1), 5.11 (*dd*, *J* = 17.4, 1.0 Hz, H-16Z), 5.09 (*dd*, *J* = 10.6, 1.4 Hz, H-16E), 4.23 (m, H-3), 3.75 (*dd*, *J* = 11.7, 4.5 Hz, H-12), 1.01, 0.99, 0.89 and 0.80 (s, 4CH_3); Data for major diol **7**: m.p. 166–169 °C; $[\alpha]_D^{25}$ −68.8° (*c* 0.6, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.82 (*dd*, *J* = 17.6, 10.9 Hz, H-15), 5.38 (*s*, H-1), 5.11 (*dd*, *J* = 17.6, 1.1 Hz, H-16Z), 5.08 (*dd*, *J* = 10.8, 1.3 Hz, H-16E), 4.23 (*m*, H-3), 3.80 (*dd*, *J* = 10.5, 5.8 Hz, H-12), 1.01, 0.99, 0.89 and 0.86 (*s*, 4 CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 150.8, 148.0, 118.8, 113.1, 72.5, 67.0, 43.1, 42.9, 41.9, 41.5, 41.1, 40.2, 39.9, 32.5, 30.4, 28.8, 28.6, 28.1, 17.7, 15.1; EIMS *m*/*z* (rel. int.): 304 (65) $[M]^+$, 286 (78), 271 (35), 204 (76), 133 (75), 121 (100), 105 (68).

3.6. Petalostigmone B (2)

White crystals; m.p. 110–114 °C; $[\alpha]_D^{25}$ −71.6° (*c* 1.0, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε) 243 (0.89) nm; IR ν_{max} ($CHCl_3$) 2930, 1704, 1673, 1291 cm^{-1}; ^{1}H and ^{13}C NMR (see Tables 1 and 2); HREIMS *m*/*z* 300.2089 for $C_{20}H_{28}O_2$ (calcd. 300.2089); EIMS *m*/*z* (rel. int.): 300 $[M]^+$ (35), 285 (20), 232 (100), 217 (16), 205 (29), 189 (41), 166 (34), 148 (53), 107 (98), 91 (67).

3.7. Petalostigmone C (3)

Needle crystals; m.p. 180–184 °C; $[\alpha]_D^{25}$ +12.0° (*c* 1.0, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε) 241 (1.3) nm; IR ν_{max} ($CHCl_3$) 3417, 3014, 2936, 2869, 1656, 1615, 1385, 1215 cm^{-1}; ^{1}H and ^{13}C NMR: see Tables 1 and 2; HREIMS *m*/*z* 300.2084 for $C_{20}H_{28}O_2$ (calcd. 300.2089); EIMS *m*/*z* (rel. int.): 300 $[M]^+$ (46), 285 (11), 272 (21), 257 (37), 232 (21), 217 (13), 199 (19), 145 (39), 135 (100), 121 (38), 107 (38), 91 (63).

3.8. Pubescenone (4)

Colorless needles; m.p. 145–148 °C; $[\alpha]_D^{25}$ +73.6° (*c* 1.1, $CHCl_3$); UV (CH_3OH) λ_{max} (log ε) 242 (0.78), 230 (0.34) nm; IR ν_{max} ($CHCl_3$) 2968, 2932, 1754, 1705 cm^{-1}; ^{1}H and ^{13}C NMR (see Tables 1 and 2); HREIMS *m*/*z* 302.1886 for $C_{19}H_{26}O_3$ (calcd. 302.1882); EIMS *m*/*z* (rel. int.): 302 $[M]^+$ (40), 287 (17), 257 (13), 205 (25), 192 (28), 149 (38), 134 (65), 107 (100), 95 (65); Crystal data: Orthorhombic crystals, $C_{19}H_{26}O_3$, mol. wt. = 302.40, crystal size $0.54 \times 0.07 \times 0.06$ mm^3, space group P212121.

3.9. In vitro L1210 mouse leukemia assay

The experimental samples were made up in MeOH solution, and the measured amounts were dried down in 18 wells of 24-well plates, the other six wells being reserved as negative controls. Generally amounts sufficient to make final concentrations of 50, 25, 10, 5.0, 2.5 and 1.0 μg/mL of each sample were added to the experimental wells. After the solvent had evaporated, one thousand L1210 cells, taken from a flask in exponential growth were added to each well in 1 mL of minimal essential medium supplemented with 10% bovine serum. The plates were incubated at 37 °C with 5% CO_2 (for about 72 h) until there were 8000 cells in the control wells, at which time the numbers of cells in the experimental wells was estimated by visual observation under an inverted optics, phase-contrast microscope, and inhibition, the difference between 8000 and the numbers present in the experimental wells, was expressed as a percent of 8000.

3.10. In vitro HEPAlclc7 murine liver bioassay

The assay was done as described previously (Schmidt et al., 2004).

Crystallographic data for the structures reported in this paper (**1** and **4**) have been deposited with the Cambridge Crystallographic Data Center as supplementary publication CCDC 269094 and 269095. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [Fax: (+44) 1223 336 033; or email: deposit@ccdc.cam.ac.uk].

Acknowledgements

This work was supported by the National Institutes of Health Grants GM 54063 to K.L. Rinehart and GM 13956 to R.M. Coates. The authors thank Dr. Scott Wilson for help with the X-ray crystal analyses and Dr. Barbra Schmidt for the murine liver bioassays. Y. Jin thanks the University of Illinois for fellowship assistance.

Appendix A. Supplementary data

General experimental information and instrumentation; preparative procedures for diols **6** and **7**, PCC oxidation of

1, and *O*-methylation of **5**; physical and spectral data for **5**; and crystal data for the X-ray structure determinations of **1** and **4**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2005.09.026.

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