Journal of Natural Products
Vol. 48, No. 3, pp. 440-445, May-Jun 1985

ANTINEOPLASTIC AND PISCICIDAL 1-ALKYLDAPHNANE ORTHOESTERS FROM *PIMELEA* SPECIES

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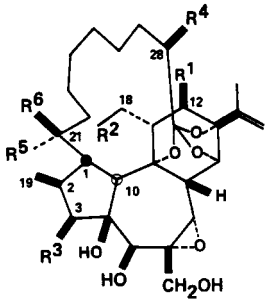
ABSTRACT.—Five biologically active daphnane orthoesters have been isolated from *Pimelea* species (Thymelaeaceae). Four of these possessing antineoplastic activity against in vivo murine P-388 lymphocytic leukemia are gnidimacrin (**1**) from *P. ligustrina*, simpleximacrin (**2**) from *P. simplex*, linimacrin d (**3**) and Pimelea factor P_3 (**4**) from *P. linifolia*. Linimacrin c (**5**) from *P. linifolia* and the other four compounds showed piscicidal activity. Gnidimacrin (**1**) and Pimelea factor P_3 (**4**) have previously been isolated from other members of the Thymelaeaceae. Simpleximacrin (**2**) and linimacrins c (**5**) and d (**3**) are new compounds.

Antineoplastic activity has been reported for extracts of *Pimelea linifolia* Sm. (1,2), *Pimelea ligustrina* Labill. (1,2), and *Pimelea simplex* F. Muell. (2,3), which grow in New South Wales, Australia. In the search for constituents responsible for this activity, five compounds have been isolated in addition to linifolins a (**6**) and b (**7**) reported previously (1). The term linifolin has been used for unrelated compounds (4), so we wish to change the name linifolin a to linimacrin a. Linifolin b should be known as Pimelea factor P_2, which was also isolated from *Pimelea prostrata* by Zayed *et al.* (5,6). The potent antineoplastic activity in P-388 lymphocytic leukemia (3PS) of *P. ligustrina* is mainly due to gnidimacrin (**1**), which was previously isolated from *Gnidia subcordata* (Thymelaeaceae) (7). Additional nmr spectral details of gnidimacrin are now presented. A new compound, simpleximacrin (**2**), which is an acetoxy derivative of gnidimacrin, is mainly responsible for the high 3PS activity (3) of *P. simplex*.

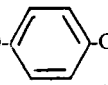
Isolation of the daphnane orthoesters was achieved by open column chromatography on silica gel of the $CHCl_3$ extract of the relevant plant, followed by preparative tlc and hplc on a reverse phase C-18 column using MeOH-H_2O mixtures as the mobile phase. Fractionation was monitored by piscicidal and 3PS activity (8). The piscicidal assay was based on those of Kupchan *et al.* (9), Sakata *et al.* (10), and Gersdorff (11), but *Gambusia affinis* fish were used. These small, hardy fish are readily available in streams worldwide.

Gnidimacrin (**1**) isolated from *P. ligustrina* appeared to be identical with the compound isolated from *G. subcordata*, by comparison of all spectral data with those obtained by Kupchan *et al.* (12). More detailed analysis of the ^{1}H-nmr spectra of gnidimacrin isolated from *P. ligustrina* has identified signals for the three methyl groups as 1.78 (s) 17-H_3, 1.14 (d, J=6Hz) 19-H_3 and 1.09 ppm (d, J=6Hz) 30-H_3. A ^{13}C-nmr spectrum was measured at 50.309 MHz in $CDCl_3$, and assignments were made using a distortionless enhancement by polarization transfer (DEPT) spectrum. The high 3PS activity for gnidimacrin (7) was confirmed (T/C 180% at dose 12-16 μg/kg).

Simpleximacrin (**2**) showed a weak molecular ion at *m/z* 832 consistent with a molecular formula of $C_{46}H_{56}O_{14}$. The ^{1}H-nmr spectrum was very similar to gnidimacrin except in the aromatic region and the signal assigned to the acetate methyl at 2.07 ppm. Decoupling of protons in the aromatic region showed that the doublets at 7.5 ppm and 7.4 ppm were coupled to the doublets 8.22 and 8.10 ppm, respectively. These findings are consistent with the presence of an acetoxy group in the *para* position of one of the benzene rings. The signal at 8.22 ppm arose from protons *meta* to the acetoxy group and the signal at 7.5 ppm from the protons *ortho* to the acetoxy group. It was not possible to determine which of the two benzene rings bore the acetoxy group. Simpleximacrin was highly active in 3PS (T/C 208% at dose 25 μg/kg).



1 $R^1=R^6=H$, $R^2=R^3=OCOC_6H_5$, $R^4=OH$, $R^5=CH_3$

2 $R^1=R^6=H$, R^2 / R^3 $=OCOC_6H_5$, OCO-  -OAc, $R^4=OH$, $R^5=CH_3$

3 $R^1=OAc$, $R^2=R^4=R^6=H$, $R^3=OCOC_6H_5$, $R^5=CH_3$

4 $R^1=R^2=R^4=R^6=H$, $R^3=OCOC_6H_5$, $R^5=CH_3$

5 $R^1=R^2=R^5=H$, $R^3=OCOC_6H_5$, $R^4=OH$, $R^6=CH_3$

6 $R^1=OAc$, $R^2=R^4=R^5=H$, $R^3=OCOC_6H_5$, $R^6=CH_3$

7 $R^1=R^2=R^4=R^5=H$, $R^3=OCOC_6H_5$, $R^6=CH_3$

8 $R^1=R^2=R^6=H$, $R^3=OCOC_6H_5$, $R^4=OH$, $R^5=CH_3$

Linimacrin c (**5**) differs from gnidimacrin in the presence of a C-18 methyl group in place of the C-18 methylene benzoyloxy group of gnidimacrin. The ^{1}H-nmr spectrum of linimacrin c differs from that published for kraussianin (13) in the position of the signals for the three methyl doublets and also the 3-H doublet. On the basis of the ^{1}H-nmr results, it is proposed that linimacrin c and kraussianin are epimeric at C-21. Linimacrin c displayed lower 3PS activity than kraussianin (linimacrin c T/C 122% at dose 360 μg/kg).

Linimacrin d (**3**) appears to be epimeric at C-21 with linimacrin a (**6**). An isomer of linimacrin a, dircin, with an acetoxy group at C-28 of the macrocyclic chain has been reported (14). The ^{1}H-nmr doublet at 5.28 ppm which was assigned to the C-28 methine proton in dircin was absent in linimacrins a and d. The signal for 12-H near 5.0 ppm indicated that this is the site of attachment of the acetoxy group in linimacrins a and d. Downfield shifts of the protons attached to C7, C8, C14, and C18 also support the assignment of a 12β-acetate. The acetate methyl signals in linimacrins a and d are further upfield (1.79 and 1.81 ppm) than expected. However, this could be due to shielding by the aromatic ring of the β-benzoate ester moiety on C-3. Similar shifts have been observed for acetate methyl groups in 12-*O*-deacetyl,12-*O*-benzoyl pteroidine (15), and euphornin (16), each of which also have benzoate ester groups. Linimacrin a (**6**) was inactive in 3PS at the highest dose tested (T/C 107% at 100 μg/kg), but linimacrin d showed weak activity (T/C 132% at 300 μg/kg). Pimelea factor P_3 (**4**) was active in 3PS (T/C 164% at 300 μg/kg). Antineoplastic activity of this diterpene has not previously been reported. All the compounds reported were lethal within 24 h to *G. affinis* fish at doses of 30-60 μg/liter.

Zayed *et al.* reported (6) the presence of Pimelea factors P_2 (**7**) and P_3 (**4**) in *P. prostrata* and proposed that these compounds are epimeric at C-21. These compounds have been isolated by us from *P. linifolia*. Linimacrins a (**6**) and d (**3**) also appear to be epimeric at C-21. Based on ^{1}H-nmr and mass spectral evidence, linimacrin c (**5**) is probably epimeric with kraussianin (**8**) isolated from *Gnidia kraussiana* (13). Pettit and co-workers (17) have unequivocally established the chemical shift of the 18-methyl group in the ^{1}H-nmr spectrum of Pimelea factor P_2 (**7**) as 1.424 ppm, instead of the previous assignment by Zayed *et al.* (5) of that resonance to the 30-methyl group. Our decoupling experiments support these findings on Pimelea factor P_2 and the other com-

pounds in this series. It would therefore appear that the assignments for the 18-methyl and 30-methyl protons in kraussianin (13) should be reversed. Borris and Cordell attribute a 21*R* configuration to kraussianin based on their assignment of the 30-methyl resonance. We agree that kraussianin probably has a 21*R* configuration, but we reach this conclusion from our assignments of 19-methyl ^{1}H-nmr resonances (see below).

The configuration at C-21 in this series of epimers was arrived at after our examination of molecular models and analysis of ^{1}H-nmr spectra. Table 1 gives data for the ^{1}H-nmr spectral assignments of each of these daphnane orthoesters. The assignments were made using difference decoupling, square wave decoupling, and homonuclear two dimensional coupling spectra. Examination of a model of gnidimacrin based on the structure obtained by Kupchan *et al.* (7) from X-ray crystallography, in which the configuration at C-21 is *R*, reveals the 30-methyl group (attached to C-21) is on the α-side of the molecule. Such a configuration could result in shielding of the 10-H and 3-H α-protons. In the 21*S* configuration, the 19-methyl protons and 30-methyl protons could experience mutual shielding resulting in an upfield shift of their signals. The ^{1}H-nmr spectra in Table 1 show that the 3-H and 10-H signals of linimacrin d (**3**) and Pimelea factor P_3 (**4**) are upfield relative to those of linimacrin a (**6**) and Pimelea factor P_2 (**7**), suggesting a 21*R* configuration for the former. The 19- and 30-methyl signals of linimacrin a and Pimelea factor P_2 are upfield relative to those of linimacrin d and Pimelea factor P_3 which supports the conclusion that linimacrin a and Pimelea factor P_2 are 21*S*. At an early stage in the investigation of structures of compounds in this series, Zayed *et al.* (5) attributed a 21*R* configuration to Pimelea factor P_2. It is more difficult to determine the structures of linimacrin c and kraussianin from their ^{1}H-nmr spectra as such spectra were measured at greatly different frequencies, and smaller differences in chemical shift may be important. The differences between chemical shifts of corresponding protons do not show such definite shielding patterns. Borris and Cordell (13) have proposed that kraussianin is 21*R*. Since the 19-methyl signal in linimacrin c is significantly upfield of the corresponding signal for kraussianin, it is consistent with this analysis to assign linimacrin c as the 21*S* epimer.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The uv spectra were obtained with a Varian Techtron model 635 spectrophotometer and the ir spectra with a Perkin-Elmer model 580 spectrophotometer. ^{1}H- and ^{13}C-nmr spectra were recorded in $CDCl_3$ with a Varian XL-200 instrument using TMS as an internal standard. Mass spectra were obtained with an A.E.I. MS 12 at 70eV. High resolution mass spectra were recorded on an A.E.I. MS 902 instrument. Hplc was performed on a semipreparative scale on a Waters Associates model 6000A instrument using a C18 μ Bondapak 30 cm column and eluting with MeOH-H_2O (9:1). Tlc was performed on Merck Kieselgel $60F_{254}$ pre-coated aluminium sheets and preparative tlc on glass plates using Merck Kieselgel $60PF_{254}$ with layers 2mm thick.

PLANT MATERIAL.—*P. linifolia* was collected at Cattai, N.S.W. Australia, in November 1978. *P. ligustrina* was collected at Kiama, N.S.W., in December 1978. *P. simplex* was collected at Goodooga, N.S.W., in July 1982. All species were identified by Dr. S. Threlfall, John Ray Herbarium, University of Sydney, N.S.W. Australia. Specimen vouchers located at Macquarie University Herbarium, N.S.W., Australia.

PISCICIDAL ACTIVITY.—*G. affinis* fish collected in local streams, averaging 175 mg in weight and 2.8 cm in length were employed in this assay. Test containers were beakers containing 150 ml of aerated H_2O. Three fish which had not been fed for 24 h were used in each test and in the controls. Test materials were dissolved in Me_2CO and 0.5 ml or less of the solution were used in each test. Control fish were given the same volume of Me_2CO as the test fish. A sample was considered lethal if all three test fish died within 24 h and nonlethal if some or all of the fish survived for 24 h. In this test, the standard fish toxin, rotenone, was lethal at a concentration of 60 μg/liter.

EXTRACTION AND FRACTIONATION OF PLANT MATERIAL.—Stems and leaves of each species were

TABLE 1. ^{1}H-nmr (200 MHz) Assignments of Compounds from *Pimelea* Species[a]

H	Compound						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1	1.8			2.36			2.35
2	1.8		1.8	1.8	1.84	1.9	1.84
3	4.94d,J=6	4.89d	4.89d,J=6	4.90d,J=5	4.90d,J=4.4	5.04d,J=6	5.05d,J=4.5
5	4.06s	4.05s	4.09s	4.06s	4.06s	4.10s	4.14s
7	3.37s	3.34s	3.45s	3.34s	3.35s	3.43s	3.33s
8	3.01d,J=2.5	3.03d,J=2.5	3.54d,J=2.5	2.94 d,J=2.5	2.98d,J=2.5	3.48d,J=2.5	2.87d,J=2
10	2.93d,J=11		2.95d,J=12	2.90d,J=12	2.90d,J=12	3.11d,J=12	3.08d,J=11
11	2.6		2.4	2.5		2.5	2.6
12			4.97s			4.93s	
14	4.36d,J=2.5	4.35d,J=2.5	4.58d,J=2.5	4.22d,J=2.5	4.34d,J=2.5	4.6d,J=2.5	4.26d,J=2
16	5.17	5.12	5.03	4.96	5.15	4.96	4.94
	4.92	4.89	4.90	4.87	4.90	4.88	4.85
$17H_3$	1.78s	1.73s	1.78s	1.72s	1.77s	1.77s	1.71s
18	4.90	4.89	1.44d,J=7	1.30d,J=6	1.30d,J=7	1.52d,J=6	1.42d,J=7
	4.36	4.34					
$19H_3$	1.14d,J=6	1.11d,J=6	1.11d,J=6	1.09d,J=6	1.10d,J=7	1.03d,J=6	1.03d,J=6
$20H_2$	3.83	3.82	3.88	3.83	3.86	3.84	3.85
21	2.35		2.4	2.5	2.4		2.6
22	1.9						1.4
$30H_3$	1.09d,J=6	1.03d,J=6	0.99d,J=6	0.99d,J=6	0.98d,J=7	0.84d,J=6	0.84d,J=6
OH	2.8	2.93	2.70	2.75	2.77		
$OCOCH_3$		2.07s	1.81s			1.79s	
OCOPh	7.3-8.2 10H	7.3-8.3 9H	7.4-8.1 5H	7.4-8.1 5H	7.4-8.1 5H	7.4-8.0 5H	7.4-8.1 5H

[a]Assignments (ppm) relative to TMS, measured in $CDCl_3$ solution. J values are in Hz.

extracted with EtOH. The EtOH extract was evaporated to dryness and partitioned between $CHCl_3$ and H_2O. The 3PS activity was concentrated in the $CHCl_3$ layer. The $CHCl_3$ extract was evaporated, and the resulting material was chromatographed on silica gel (Merck TLC grade) packed in a Büchner funnel. Elution under reduced pressure with the following series of solvents gave five fractions: C_6H_6, C_6H_6-EtOAc (9:1), C_6H_6-EtOAc (6:4), EtOAc, and MeOH. In each case, the 3PS activity was greatest in the fractions eluted by C_6H_6-EtOAc (6:4) and EtOAc. The active fractions were separated further by preparative tlc and pure compounds were isolated by reverse phase hplc eluting with MeOH-H_2O (9:1) mixture.

Gnidimacrin (**1**): (1×10^{-3}% of dry weight of *P. ligustrina*); isolated from EtOAc fraction by hplc (eluted with 25 ml); tlc Rf 0.37 (EtOAc); ir ν max (thin film) 3550, 1720, 1645 cm^{-1}; uv λ max (MeOH) 229 nm (log ϵ 4.27); ms *m/z* (rel. int.) 774 (M^+, 0.2%), 756 (0.2), 743 (0.05), 725 (0.5), 670 (0.2), 652 (0.3), 122 (24), and 105 (100); ^{13}C nmr ($CDCl_3$) ppm 14.64q (C-19), 18.12q (C-30), 19.06q (C-17), 22.47t, 23.00t, 23.64t, 23.94t, 25.20t, 27.29d (C-21), 28.48t, 36.69d (C-11), 37.43d (C-1), 41.17d (C-10), 48.10d (C-8), 49.33d (C-2), 63.52d (C-7), 65.86t (C-20), 67.56t (C-18), 70.68d (C-28), 73.74d (C-5), 81.38d (C-3), 82.68d (C-14), 111.87t (C-16), 128.43d, and 128.57d (aromatic *meta*), 129.63d and 130.15d (aromatic *ortho*), 132.97d, and 133.54d (aromatic *para*).

Simpleximacrin (**2**): (3×10^{-4}% of dry weight of *P. simplex*); isolated from EtOAc fraction by hplc (eluted with 27 ml); tlc Rf 0.36 (hexane-Me_2CO, 1:1); ir ν max (thin film) 1740, 1720 cm^{-1}; uv λ max (MeOH) 282 (sh. log ϵ 3.48), 273 (3.59), 268 (3.61), and 229 nm (5.23); ms *m/z* (rel. int.) 832 (M^+, 0.25%), 814 (0.25), 801 (0.1), 783 (0.5), 710 (0.6), 692 (0.6), 650 (1), 105 (38), and 43 (100).

Linimacrin d (**3**): (4×10^{-5}% of dry weight of *P. linifolia*); isolated from C_6H_6-EtOAc (6:4) fraction by hplc (eluted with 36 ml); tlc Rf 0.51 (EtOAc); ir ν max (thin film) 1743, 1718 cm^{-1}; uv λ max (MeOH) 276 (log ϵ 2.62), 263 (2.87), and 230 nm (4.22); ms *m/z* (rel. int.) 696 (M^+, 3%), 678 (1), 665 (1), 636 (4), 607 (2), 595 (3), 589 (2), and 105 (100); cims (hydrogen gas) $M^+ + H$697.3567 ($C_{39}H_{53}O_{11}$ requires 697.3587).

Pimelea factor P_3 (**4**): (1.4×10^{-4}% of *P. linifolia*); isolated from C_6H_6-EtOAc (6:4) fraction by hplc (eluted with 41 ml) tlc Rf 0.53 (EtOAc); ir ν max (thin film) 1718 cm^{-1}; uv λ max (MeOH) 275 (log ϵ 2.94), 268 (2.95), and 230 nm (4.13); ms *m/z* (rel. int.) 638 (M^+, 5%), 620 (1), 607 (5), 595 (10), 589 (4), and 105 (100); M^+ 638.3468 ($C_{37}H_{50}O_9$ requires 638.3453).

Linimacrin c (**5**): (1.3×10^{-4}% of dry weight of *P. linifolia*); isolated from EtOAc fraction by hplc (eluted with 26 ml); tlc Rf 0.33 (hexane-Me_2CO, 1:1); ir ν max (thin film) 1720 cm^{-1}; uv λ max (MeOH) 279 (log ϵ 3.34), 273 (3.31), and 229 nm (3.82); ms *m/z* (rel. int.) 654 (M^+, 2%), 623 (2), 605 (2), and 105 (100); M^+ 654.3433 ($C_{37}H_{50}O_{10}$ requires 654.3403).

ACKNOWLEDGMENTS

This work was supported in part by the Australian Research Grants Committee, The Children's Leukaemia and Cancer Foundation, William S. Merrell Pty. Ltd., and David Bull Laboratories Pty. Ltd.

The authors wish to thank Drs. M. Batley, I. Brown, and S. Threlfall; Messrs. A. Blofeld, A. Brown, F. Geiser, A. Gooley, D. Nelson, P. Pembroke, A. Tseng, and K. Whitehurst for special assistance.

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Received 12 November 1984