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THE CYTOTOXIC NORDITERPENE DILACTONES OF *PODOCARPUS MILANJIANUS* AND *PODOCARPUS SELLOWII**

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Key Word Index—*Podocarpus milanjianus* and *Podocarpus sellowii*; Podocarpaceae; bark; norditerpene dilactones; 9KB cytotoxicity; structural analysis; nagilactone F; nagilactone G.

Abstract—The cytotoxic norditerpene dilactones nagilactone F and its new congener nagilactone G have been isolated from the bark constituents of *Podocarpus milanjianus* and *Podocarpus sellowii*. The diterpenes totarol, 19-oxototarol and macrophyllic acid were also isolated.

INTRODUCTION

The genus *Podocarpus* (family Podocarpaceae) is well known to natural product chemists as a rich source of terpenic substances. The discoveries of the growth retardant [1–4] and insecticidal properties [5–7] attributable to constituents of several *Podocarpus species* has led to the isolation of a number of nor- and bisnorditerpene dilactones. An excellent review is available concerning the biogenesis, biological activity, chemistry and spectroscopy of this unique class of compounds [8]. There are a number of recent reports on the isolation of new members of the norditerpene dilactone group [9–12].

Additionally, two norditerpene dilactones have been assayed in animal tumor systems and found to possess antitumor activity, although only a few members of this class have been evaluated. An activity-directed isolation of *P. gracilior* led to the isolation of podolide (**1**, NSC 238978), which had marginal activity in P388 and was cytotoxic in 9KB [13]. In addition, nagilactone C (**2**, NSC 117884) had significant activity against cultured Yoshida sarcoma cells and P388 (T/C 145% at 40 mg/kg) [11, 14]. Other compounds tested in this study were also determined to be cytotoxic in the 10^{-3} to 10^{-4} mM range against the Yoshida sarcoma culture (5×10^4 cells/ml) [14].

In the continued search for new effective antitumor agents from plants, we have tested the chloroform-soluble fraction of the various plant parts of *P. milanjianus* and have found *in vivo* P388 and *in vitro* 9KB antitumor activity [15] to be concentrated in the stem bark (Table 1). An activity-directed isolation has revealed that the cytotoxic components differ from those responsible for the P388 activity. The isolation, purification and characterization of the cytotoxic components are, in part, reported here. In a related study of the bark constituents of *P. sellowii*, identical norditerpene dilactones were also obtained but from the benzene-soluble fraction of the bark.

Table 1. 9KB and P388 activities [15] of the $CHCl_3$-solubles of *Podocarpus milanjianus*

Plant part	9KB (ED_{50}, μg/ml)	P388 (% T/C at mg/kg)
Leaf	16	Toxic at 400
		147 at 200
		147 at 100
Twig	16	126 at 200
Stem bark	0.22	Toxic at 400
		Toxic at 200
		163 at 100
Wood stem	20	138 at 200
Root	7.1	133 at 200

RESULTS AND DISCUSSION

P. sellowii, a tree up to 15 m tall, is distributed throughout southern Brazil. The bark of this conifer was collected and fractionated as indicated in the Experimental yielding totarol [16], mixtures of long-chain hydrocarbons and fatty acids, macrophyllic acid [17] and two novel substances A and B.

In a preliminary study, the 95% EtOH extract from various plant parts of *P. milanjianus* were partitioned between water and $CHCl_3$. Assay of the chloroform soluble fractions against 9KB and P388 indicated that the stem bark extracts were most active and further

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‖ In partial fulfillment of dissertation requirements, "The Chemical Constituents of Two *Podocarpus* sp.", Indiana University, Bloomington, IN 47401, U.S.A. (Sept. 1969), *Diss. Abstr. Int. B* **30**, 5444 (1970), *Chem. Abstr.* **74**, 28303r (1971).

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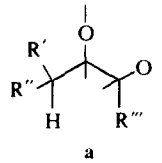
fractionation was carried out using this plant part as outlined in the Experimental. PLC of the active chloroform-soluble fraction (P388–200% T/C at 400 mg/kg; 9KB–2.2 μg/ml) on Si gel provided a band at R_f 0.75 with an ED_{50} of 0.62 μg/ml. Based on this, the $CHCl_3$-solubles were chromatographed on Si gel. A crystalline compound was obtained by elution with benzene–hexane (1:1) and was identified as totarol [12] which had an ED_{50} of 4.9 μg/ml. Subsequently, 19-oxototarol [18] was eluted with $CHCl_3$–MeOH (99:1). Elution with $CHCl_3$–MeOH (98:2) provided two fractions with ED_{50} values of 0.22 and 2.1 μg/ml, respectively. These fractions contained components analogous to the active PLC band above, and were therefore combined for low pressure chromatography on Si gel. On chromatography, a fraction was eluted with $CHCl_3$–benzene (95:5) which possessed activity (0.35 μg/ml). Recrystallization of this active fraction provided approximately 150 mg of a solid having an ED_{50} value of 0.017 μg/ml against the 9KB cell culture system. MS indicated this fraction to be essentially a mixture of four related compounds [M^+ *m/e* 316 (A), 332 (B), 314 (C) and 330 (D)]. An initial separation by column chromatography on C_{18} Si gel (reversed-phase) gave a fraction (75%) containing A and B and a fraction (25%) containing C and D. Further separation of components A and B was effected by multiple development PLC on Si gel to afford compounds identical to those (A and B) obtained from *P. sellowii*. Both compounds had ED_{50} values in the 10^{-2} range vs 9KB cells.

These compounds appeared to be related norditerpene dilactones by analogy to published data [8]. Substance A, mp 296–298°, had a MW of 332.162 consistent with a molecular formula of $C_{19}H_{24}O_5$. Its ^1H NMR spectrum exhibited four methyl signals at δ 1.10 (6H), 1.15 and 1.28, and five single-proton signals at 1.85, 3.95, 4.40, 4.91 and 5.95. Decoupling experiments demonstrated that the two methyl-doublet at 1.10 was due to an isopropyl group, since irradiation in the methine region caused collapse of these two peaks to a singlet. Irradiation in this same region caused collapse of the 4.91 doublet of doublets and of the 4.40 doublet to a singlet. The observation that no single irradiating frequency was able to cause simultaneous maximum collapse of the isopropyl methyls, the single hydrogen doublet of doublets, and the single hydrogen doublet or any combination thereof indicated that these moieties were not coupled to the same proton. Irradiation of the 4.91 doublet of doublets collapsed the 3.95 doublet to a singlet demonstrating that these two protons were coupled. These results indicated the presence of fragments **a** and **b** in substance A, assuming that the single hydrogen multiplets arose from protons attached to carbon atoms singly bonded with oxygen atoms.



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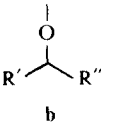


b

The IR spectrum of substance A showed carbonyl absorption at 1776 and 1706 cm^{-1} suggestive of the presence of a 5-membered ring lactone and a conjugated unsaturated 6-membered ring lactone. The UV absorption, λ_{max}^{EtOH} 218 (ε = 10000) was also indicative of an α,β-unsaturated lactone. These spectral data and the close correlation with the chemical shifts and coupling constants for protons on C-5, C-6, C-7 and C-11 by Hayashi *et al.* [3] and Ito *et al.* [19] for the nagilactones and inumakilactone from *P. nagi* and *P. macrophyllus*, in addition to a comparison to reference podolide [13], revealed the stereochemistry and structure for substance A, a previously unreported compound for which we propose the name nagilactone G (**3**).

Substance B, mp 223°, had a MW of 316.165 consistent with a molecular formula of $C_{19}H_{24}O_4$. The difference between this compound and nagilactone G (**3**) was one less oxygen atom without a change in the degree of unsaturation, suggesting that substance B was nagilactone F (**4**) [3]. The spectral data for B corresponded to those reported by Hayashi *et al.* [3] for nagilactone F.

Sellowin A, B and C have been previously isolated from the leaves and wood of *P. sellowii* [20], whereas *P. milanjianus*, a timber tree representing the little studied African *Podocarpus*, has previously yielded totarol, 19-oxototarol and 19-hydroxytotarol [21] and kaurene and isokaurene [22] but no norditerpene dilactones. The isolation and characterization of nagilactone F and its new congener nagilactone G is of note due to their significant activity against the 9KB nasopharyngeal cell culture system (10^{-2} μg/ml). Certain Florisil column fractions from *P. milanjianus* have been determined to be even more active (158% T/C at 35 mg/kg and 158% T/C at 7.9 mg/kg) than that activity previously demonstrated by podolide or nagilactone C and further work is underway to isolate these active components and to determine the structures of the compounds C and D from *P. milanjianus*.

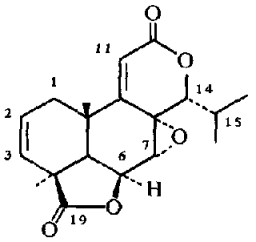
EXPERIMENTAL

All mps are uncorr. The IR spectra were taken in KBr. ^1H NMR data were obtained in $CDCl_3$, shifts are reported in δ (ppm) units and coupling constants in Hz. For absorption TLC and PLC, Si gel GF-254 and PF-254 prepared plates (E. Merck, Darmstadt, Germany) were utilized. Reversed-phase partition TLC was performed on Whatman KC_{18} TLC plates with fluorescent indicator. C_{18} reversed-phase Si gel was prepared [23] for column chromatography using Quantum Industry's LP-10 silica gel (10–20 μ).

Podocarpus sellowii *collection.* The bark (6.7 kg) of *P. sellowii* Klotzsch was collected in the vicinity of Curitiba, Parana, Brazil in April, 1967.

Podocarpus sellowii *extraction.* Coarsely ground plant material (6.7 kg) was extracted in 500 g portions with 4 l. C_6H_6 for 24 hr in a Soxhlet apparatus. Solvent removal *in vacuo* yielded 61.2 g (0.90% dry wt) of a dark brown viscous oil. Extraction of a 1.5 l. petrol soln of the oil with three 500 ml portions of 5% aq. KOH, drying the organic layer with dry $MgSO_4$, and removing the solvent *in vacuo*, yielded a light yellow oil (9.6 g) consisting of the neutral components. Acidification (pH 5) of the basic aq. layer with 0.1 N HCl, extraction with three 500 ml portions of $CHCl_3$, drying over $MgSO_4$ and removal of solvent *in vacuo* yielded 41.3 g of brown gum consisting of the acidic components.

Podocarpus sellowii *isolation procedure.* Upon standing overnight, the neutral oil mixture deposited 850 mg of colourless needles which were removed by filtration. Si gel (120 g, 50–200 mesh) CC of the oil and elution with Et_2O–petrol yielded 596 mg of material having the spectral characteristics of a mixture of long-chain hydrocarbons and 40 mg of material,



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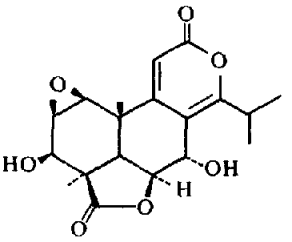

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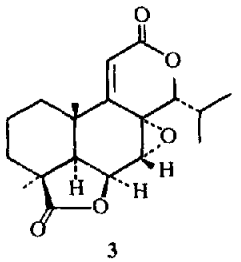

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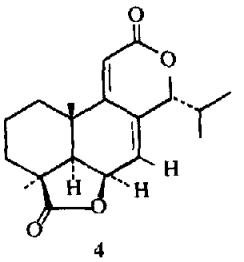

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which upon rechromatography, produced 23 mg of a crystalline substance, mp 131–2°, $[\alpha]_D$ +41° (c = 0.09, MeOH) identical in all respects with an authentic sample of totarol [16]. Continued elution with Et_2O produced 2.0 g of material having the spectral characteristics of long-chain fatty acids. Neither the hydrocarbons nor the fatty acids were investigated further. The MS of the above deposited needles, from the neutral oil mixture, with 10 eV ionization, produced peaks at m/e 316 and 332 indicating the material to be a mixture of at least two compounds. Small quantities of two pure materials, substance A (10 mg) and B (10 mg) identified as nagilactone G and F respectively, were recovered from Si gel (200 g) CC with CH_2Cl_2 elution of 100 mg of the A–B mixture (the intermediate fractions were identical with the starting mixture). Chromatography of the acidic components obtained, as described, over Si gel (3.2 kg) and C_6H_6 elution yielded 10.4 g of material having the spectral characteristics of a mixture of long-chain fatty acids and was not investigated further. However, 744 mg of brown gum obtained was rechromatographed yielding 30 mg of fine needles, mp 235–8°, $[\alpha]_{25}^{D}$ +125° (c = 0.04, MeOH), MS m/e 630 (M^+), found to be identical to an authentic sample of macrophyllic acid [17]. A 343 mg portion of the brown gum yielded, on rechromatography, 27 mg of a mixture of substances A and B.

Podocarpus milanjianus *collection*. The stem bark (29 kg) of *P. milanjianus* Rendle was collected in Kenya in July, 1973 under the direction of Dr. R. E. Perdue, Jr., U.S.D.A.

Podocarpus milanjianus *extraction and fractionation*. Ground plant material (10 kg) was wet-packed into a large copper percolator and extracted with 95% EtOH (91 l.) to yield 559.0 g (5.6% of dry wt) of residue. The 95% EtOH-solubles were partitioned between H_2O and $CHCl_3$ yielding, respectively, 202.5 and 165.3 g of residue in addition to an interface residue (83.2 g). The $CHCl_3$-solubles were subsequently partitioned between 10% aq. MeOH and petrol yielding, respectively, 95.8 and 44.7 g of residue, whereupon the 10% MeOH-solubles were further partitioned between 40% aq. MeOH and $CHCl_3$ to yield 13.1 and 66.5 g of residue, respectively. Fractions and compounds were tested against the P388 lymphocytic leukemia system in the mouse and Eagles 9KB nasopharyngeal carcinoma cell culture system according to established protocols [15]. In the P388 system, an effective response is obtained when a minimal percentage increase in the median survival time of treated animals over control animals (% T/C) results in a 130% T/C. In the 9KB cell culture system, results are expressed at that dose which inhibits growth to (ED_{50}). An ED_{50} of 4 μg/ml and an ED_{50} 20 μg/ml is considered effective for pure compounds and crude plant fractions, respectively. The P388 antitumor testing was performed by RALTECH Scientific Services, Inc., while the 9KB cytotoxicity testing of samples was performed by the Purdue Cancer Center Cell Culture Laboratory.

PLC of the $CHCl_3$-solubles (100 mg) on Si gel using MeOH–$CHCl_3$ (1:9) as the developing solvent provided 7 bands visualized with UV (R_f 0.92, 3.8 mg; R_f 0.30, 9.8 mg; R_f 0.18, 2.3 mg; R_f 0.1, 4.0 mg; and, the origin, 5.6 mg). Fractions were submitted for 9KB assay.

A further portion of the $CHCl_3$-soluble fraction (20 g) was subjected to CC on Si gel (750 g, 70–270 mesh, Machery-Nagel and Co.). The elution process involved a step-wise elution from C_6H_6–$CHCl_3$ (1:1) through various mixtures of $CHCl_3$ and MeOH. Fractions (15 ml) were combined according to TLC analysis and then submitted for testing against the 9KB cell culture system. The initial fraction (0.671 g) eluted with C_6H_6–$CHCl_3$ (1:1) was homogeneous by TLC and recrystallization from hexane provided 120 mg of a crystalline material identical in spectral characteristics to totarol [12] but with trace contamination difficult to remove. Further purification was not pursued. IR ν_{max} cm^{-1}: 3568, 3456 (b), 1595, 1490, 1380 and 1360; ^1H NMR ($CDCl_3$): δ 0.91 (3H, *s*), 1.18 (3H, *s*), 1.34 (6H, *d*, $J_{15,16(17)}$ = 7), 3.35 (1H, *heptet*), 6.52 (1H, *d*, $J_{11,12}$ = 8.5) and 7.06 (1H, *d*, $J_{11,12}$ = 8.5). Confirmation of the position of the phenolic OH group was achieved by ^1H NMR in Py-d_5 whereas a Py solvent effect (+0.69 ppm) was affected on the δ 7.06 C-12 proton. Examination of the MS for this compound indicates the $M^+ - 15$ peak to be more intense than the parent ion [24] which is characteristic of totarol. 19-Oxototarol [18] was eluted with $CHCl_3$–MeOH (99:1) in a fraction (127 mg) essentially homogeneous by TLC. The residue was triturated with hexane and recrystallized from hexane–$CHCl_3$ providing 66 mg of this diterpene with trace contamination (MS, ^1H NMR) from 19-hydroxytotarol. Further purification was not performed. UV λ_{max}^{EtOH} nm: 275 (ε = 2000), 325 sh (ε = 700); IR ν_{max} cm^{-1}: 3420, 3370, 2915, 2860, 1700, 1648, 1585 and 1280; ^1H NMR ($CDCl_3$): δ 1.06 (3H, *s*), 1.12 (3H, *s*), 1.35 (6H, *d*, $J_{15,16(17)}$ = 8), 6.59 (1H, *d*, $J_{11,12}$ = 9), 7.05 (1H, *d*, $J_{11,12}$ = 9), 9.98 (1H, *s*); MS, M^+ m/e 300.

The 9KB active column fractions (0.476 g, ED_{50} 0.22 μg/ml and 6.826 g, ED_{50} 2.1 μg/ml) were eluted from the above Si gel column with $CHCl_3$–MeOH (98:2 to 95:5) and contained components analogous (by chromatography) to those contained in the active fraction from PLC of the $CHCl_3$ extract. Based on activity, the fractions were combined for low pressure (< 50 psi) CC on Si gel (600 g, 10–20 μ, Quantum Industries). Elution was initiated with C_6H_6–$CHCl_3$ (1:9) and proceeded step-wise through mixtures of $CHCl_3$–MeOH. Fractions (100 ml) were combined according to TLC analysis and then submitted for testing. 9KB activity was concd in a fraction eluted with C_6H_6–$CHCl_3$ (5:95). Trituration of the residue (367 mg) with hexane and recrystallization from C_6H_6–hexane provided 150 mg of a crystalline material. TLC on C_{18} reversed-phase plates, utilizing MeOH–HOH–AcCN (5:3:2) as the developing solvent, revealed a lower-running major spot (R_f 0.58) and a higher-running minor spot (R_f 0.63) upon visualization. Separation of the two mixtures was accomplished by reversed-phase column chromatography on C_{18} Si gel (200 g) with the above solvent system at 80 psi. Fractions (1 ml) were collected and the eluant monitored by UV. Fraction collection was initiated when elu-

tion of the minor component mixture was first noted. Fraction A (tubes 1–10, 10.2 mg), fraction B (tubes 11–14, 6.5 mg) and fraction C (tubes 15–90, 76.3 mg) were obtained accordingly and tested for activity. Analysis of the respective fractions on C_{18} Si gel TLC plates indicated fraction A to be homogeneous, fraction B to be a mixture identical to the original mixture and fraction C to be homogeneous. No further analysis of fraction A was attempted. Fraction C (62 mg) was subsequently partially separated by multiple development PLC on 5 Si gel plates (2 mm) utilizing CH_2Cl_2 as the developing solvent. Visualization revealed a minor UV quenching band appearing above and overlapping with the major UV quenching band. The highest moving band was ascertained to be substance A (14.8 mg), whereas substance B (28.7 mg) was determined to comprise the lower band, which followed an overlapping mixture (13.8 mg).

Nagilactone G (NSC 306213, *substance A*). Crystallization of substance A (14.8 mg) was achieved with difficulty from C_6H_6–hexane, mp 296–298° (colourless needles); ^{1}H NMR ($CDCl_3$): δ 1.10 (6H, *d*, $J_{15,16(15,17)} = 7$), 1.15 (3H, *s*), 1.28 (3H, *s*), 1.85 (1H, *d*, $J_{5,6} = 5$), 3.95 (1H, *d*, $J_{6,7} = 2$), 4.40 (1H, *d*, $J_{14,15} = 5$), 4.91 (1H, *dd*, $J_{6,7} = 2$, $J_{5,6} = 5$), 5.95 (1H, *s*); UV λ_{max}^{EtOH} nm: 218 ($\varepsilon = 10000$); ORD ($c = 0.07$, MeOH), $[\alpha]_{700}^{25}$ −2.5°, $[\alpha]_{600}$ −7.5°, $[\alpha]_{589}$ −7.8°, $[\alpha]_{500}$ −15°, $[\alpha]_{400}$ −43°, $[\alpha]_{350}$ −85°, $[\alpha]_{300}$ −1160°, $[\alpha]_{280}$ −2140°, $[\alpha]_{250}$ −1950°, $[\alpha]_{220}$ −1720°; MS *m/e* (rel. int.): 332 (M^+, 3), 317 (2), 314 (3), 304 (12), 289 (15), 261 (100), 233 (36), 215 (31); IR ν_{max}^{KBr} cm^{-1}: 1776 and 1706. High resolution MS, $C_{19}H_{24}O_5$, calc.: 332.162. Found: 332.162.

Nagilactone F (NSC 251688, *substance B*). The pure solid (28.7 mg) was recrystallized from abs. MeOH, mp 223° (sharp needles) (lit. 225–226° [3]; ^{1}H NMR ($CDCl_3$): δ 0.98 (3H, *d*, $J_{15,16} = 7$), 1.17 (3H, *s*), 1.19 (3H, *d*, $J_{15,16(17)} = 6.8$), 1.35 (3H, *s*), 1.95 (1H, *d*, $J_{5,6} = 5$), 4.89 (1H, *q*, $J_{14,15(14,6)(14,7)} = 2$), 5.08 (1H, *td*, $J_{6,7(6,5)} = 5$, $J_{6,14} = 2$); 5.77 (1H, *d*, $J_{7,11} = 2$), 6.19 (1H, *dt*, $J_{6,7} = 5$, $J_{7,14(7,11)} = 2$); UV λ_{max}^{EtOH} nm: 260 ($\varepsilon = 15300$); ORD ($c = 0.18$, MeOH), $[\alpha]_{700}^{25}$ −69°, $[\alpha]_{600}$ −97°, $[\alpha]_{589}$ −111°, $[\alpha]_{500}$ −181°, $[\alpha]_{400}$ −487°, $[\alpha]_{350}$ −1040°, $[\alpha]_{300}$ −4850°, $[\alpha]_{296}$ −4900°, $[\alpha]_{250}$ −2450°, $[\alpha]_{220}$ −1500°; MS *m/e* (rel. int.): 316 (M^+, 33), 288 (14), 273 (30), 245 (100), 229 (18), 227 (23), 217 (37), 145 (32). High resolution MS, $C_{19}H_{24}O_4$, calc.: 316.167. Found: 316.165.

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