A New Glycoside Antimicrobial Agent from *Persoonia linearis* × *pinifolia*

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Received December 6, 1996[⊗]

A compound possessing antimicrobial activity, which is present in the ripening fruit of *Persoonia linearis* × *pinifolia*, has been identified as 4-hydroxyphenyl 6-*O*-[(3*R*)-3,4-dihydroxy-2-methylenebutanoyl]-*β*-D-glucopyranoside (**1**), using spectroscopic techniques. Its structure was confirmed by X-ray crystallography.

Persoonia pinifolia R. Br. (Proteaceae), commonly known as pine-leaf geebung, is a bushy tree native to the southeastern parts of Australia between 33° and 36° latitude. The first reported study of the chemistry of *P. pinifolia* was carried out by Gascoigne *et al.*[1] who examined the anthocyanin content of the fruit. During the 1940s and 1950s, Atkinson[2,3] undertook an extensive in vitro screening program for antibacterial activity of Australian native flowering plants and fungi using *Staphylococcus aureus* (golden staph) and *Salmonella typhi* (typhoid) as test organisms. Of the many hundreds of plants tested, only a handful were active against both bacteria. These included extracts of the fruit of *P. pinifolia* and two other *Persoonia* species. The *P. pinifolia* extract also showed inhibitory activity against a wide range of other pathogenic bacteria. Attempts by Atkinson to isolate and purify the antibiotic component were unsuccessful.[2,3] In the present study, we have isolated a compound from a *P. pinifolia* hybrid, which shows intense broad-spectrum antimicrobial activity, and have identified it as a new natural product closely related to the tuliposides.

The ripening fruits of *P. linearis* × *pinifolia*, a cross-hybrid of *P. pinifolia* and *P. linearis*, growing in the National Botanic Gardens, Canberra, were collected, lyophilized, macerated, and extracted with a series of solvents of increasing polarity. Both the EtOAc and the MeOH extracts were active against the bacteria *Bacillus subtilis* (Gram positive) and *Escherichia coli* (Gram negative) and the fungus *Phytophthora cinnamomi*. Bioassay-guided fractionation of the extracts led to the isolation of a single antimicrobial compound with structure **1**.

Ions at *m*/*z* 385 $[M - H]^-$ and 387/409 $[M + H/M + Na]^+$ in the negative- and positive-FABMS indicated a molecular weight of 386 Da for **1**, while from elemental analysis a molecular formula of $C_{17}H_{22}O_{10}$ could be deduced. Signals in the region of δ 3.0 to 5.0 in the ^{1}H-NMR spectrum with a characteristic anomeric proton signal at δ 4.81 (J = 8 Hz) suggested that **1** was a glycoside. Two one-proton resonances at δ 6.03 and 6.38 (J =1.5 Hz) indicated the presence of an exocyclic methylene group, while two doublets at δ 6.69 and 6.93, which integrated for four protons, were typical of a *para*-substituted benzene ring with electron-donating substituents. The ^{13}C-NMR spectrum contained readily identifiable lowfield signals for the anomeric, exocyclic methylene and aromatic carbons, as well as a signal at 167.5 ppm, which, combined with an IR absorption band at 1704 cm^{-1}, could be assigned to an α,β-unsaturated ester carbon.

In the COSY spectrum two isolated spin systems could be identified in the region containing oxygen-substituted resonances. The first was a seven-proton system with a methylene group and five methine protons, all with J values >7 Hz, supporting the presence of a β-glucopyranose ring. The downfield shift of the methylene protons by ca. 1 ppm compared with that in the unsubstituted sugar suggested that the ester group was attached to the C-6 position. The other was a three-proton ABX system of which the X proton at δ 4.61 also showed a small coupling to the exocyclic methylene protons, indicating that it was part of the C_5 ester moiety. The remaining oxygen could be assigned to the *para*-position of the aromatic ring to give a hydroquinone ether. In the long-range GHMBC spectrum of **1**, there was a correlation between the anomeric proton at δ 4.81 and C-1″ of the aromatic ring at 152.4 ppm, confirming the attachment of the hydroquinone at C-1 of the sugar. This allowed us to assign structure **1** for the antimicrobial, having a β-glucopyranose ring substituted at the C-1 and C-6 positions, with a hydroquinone ether and a 3,4-dihydroxy-2-methylenebutanoyl ester, respectively.

The absolute configuration at C-3′ in the ester side chain was determined by acid hydrolysis of **1** to give the hydroxymethylenelactone (**2**). Although identical in all other respects, its optical rotation was equal and opposite to that reported for (*S*)-3-hydroxy-2-methylene-γ-butyrolactone [(−)-tulipalin B],[4] and therefore the *R*-configuration could be assigned to **2**. The *R*-enantiomer has previously been synthesized,[5] but only recently has (+)-tulipalin B been reported as a natural product.[6] Hydroquinone and D-glucose were also identified in the acid hydrolysate.

Verification of the structure of **1** was provided by an X-ray crystal structure (Figure 1), which also confirmed the absolute stereochemistry at C-3′. After a preliminary report of this work,[7] we were contacted by a New Zealand group that had isolated a similar compound from a native New Zealand plant. Subsequent correspondence confirmed that the two compounds were identical.[8]

Compound **1** was assayed using the three test organisms at loadings of 200, 100, 50, 25, 12.5, and 6.25 μg/disk. The results showed that, after 24 h, growth inhibition of *B. subtilis* by **1** was not observed below 12.5 μg/disk, while the other two organisms, *P. cinnamomi* and *E. coli*, showed inhibition even at the lowest loading

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[⊗] Abstract published in *Advance ACS Abstracts,* May 15, 1997.

S0163-3864(97)00006-2 CCC: $14.00

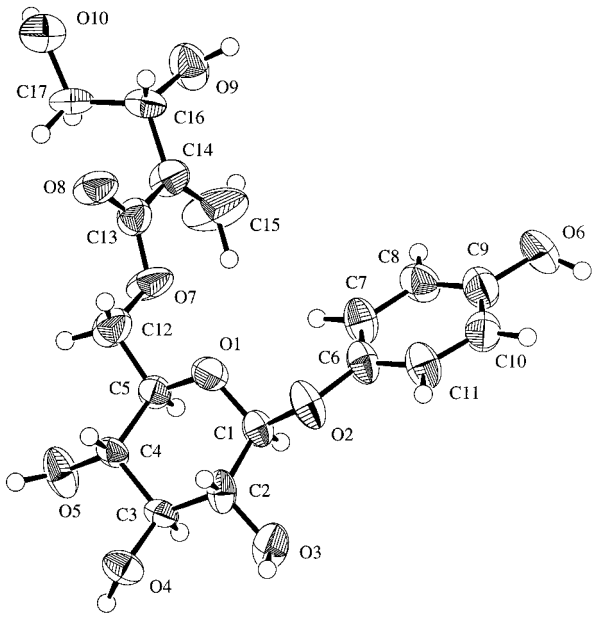
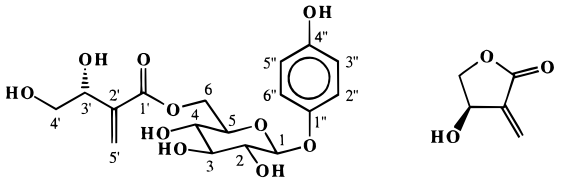


Figure 1. ORTEP drawing of **1** derived from X-ray crystallographic data.

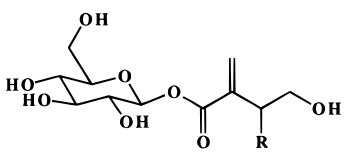
(6.25 μg/disk). This was, however, close to the minimum inhibitory concentration based on a graph of the extent of the inhibition zone versus concentration.

A number of other glucosides containing monohydroxy- and dihydroxy-2-methylenebutanoyl ester substituents at the C-1 and C-6 positions, called tuliposides, (e.g., **3** and **4**), have been isolated from several species of *Alstroemeria* (Alstromeriaceae) and *Tulipa* (Liliaceae).[4,9] Compounds **3a** and **3b** have been shown to be responsible for contact dermatitis, known as "tulip finger," which is commonly found in people working with plants from these families.[9] This has been attributed to their facile hydrolysis to give as the active compounds the α−methylenebutyric acid and its corresponding α-methylenebutyrolactone. Although the C-1 tuliposides (**3a/b**) show antibacterial activity against *B. subtilis*, the more stable C-6 tuliposides (**4a/b**) are reported to be inactive.[4] Thus, the strong antibacterial activity of **1** would appear to be related to the presence of the hydroquinone ether at C-1. On the other hand, 4-hydroxyphenyl β-D-glucopyranoside (arbutin), which is widespread in the Ericaceae, has not been reported as having antibacterial activity.

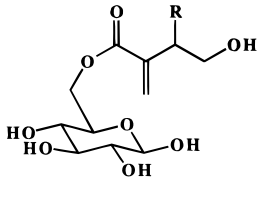


1

2



3a: R = H
b: R = OH



4a: R = H
b: R = OH

Experimental Section

General Experimental Procedures. Melting points were determined on a Reichert hot-stage apparatus and are uncorrected. Microanalyses were carried out by the ANU Microanalytical Service. IR spectra were recorded on a Perkin-Elmer 1800; and UV spectra, on a Varian Cary 5 spectrophotometer. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. ^{1}H-NMR spectra were recorded on either a Varian Gemini or a VXR-300 spectrometer at 300 MHz or on a Varian VXR-500 spectrometer at 500 MHz, using TMS as internal standard. ^{13}C-NMR spectra were recorded on either a Varian Gemini or a VXR-300 instrument at 75.5 MHz using the solvent signal as internal reference. The HMQC and GHMBC ($^nJ_{CH}$ optimized for 8 Hz) experiments were performed using standard Varian pulse sequences. LREIMS and HREIMS at 70 eV were obtained on either a VG7070F or a VG ZAB-2SEQ mass spectrometer. FAB (positive and negative) mass spectra were recorded on the VG ZAB-2SEQ instrument using a Cs ion gun. Chromatographic separations were carried out using vacuum−liquid chromatography (VLC)[10,11] on Si gel 60G (Merck). Precoated aluminum Si gel plates (Merck, Kieselgel 60 F_{254}, 0.20 mm) were used for analytical TLC.

Plant Material. The fruits of *P. linearis* × *pinifolia* were collected in mid-September 1994, from a tree growing in the National Botanic Gardens (NBG), Canberra, ACT (No. 8004240−site 23), with the permission of the NBG management.

Bioassays. The microorganisms used in the bioassays, *B. subtilis* RSC 1, *E. coli* RSC 2, and *P. cinnamomi* RSC 290 are stock test organisms continuously cultivated in the microbiological laboratory of the Research School of Chemistry. The plates for the bacterial assays were made up in batches of 10 by inoculating molten agar (100 mL, 40−45 °C) with the appropriate organism and pipeting 10 mL into sterile petri dishes. The plates were stored at 4 °C and used within 5 days of preparation. Fungal assays used a strip of inoculum to produce a straight growing front. To carry out the assay, samples of extracts were dissolved in an appropriate solvent, and aliquots equivalent to 1 mg and 0.1 mg of the dry sample were used to wet filter paper disks (5 mm). The disks were air-dried, placed on an inoculated agar plate, and incubated at 28 °C overnight. The inhibition zones were measured after 18−24 h. The solvent used to dissolve the extract was included as a blank.

Extraction and Isolation. The fruits of *P. linearis* × *pinifolia* (930 g) were lyophilized, and the dried material (220 g) was macerated with heptane (1 L). The pulp was left for 24 h, filtered, and the maceration procedure repeated 4 times. The pulp was then extracted at room temperature consecutively with CH_2Cl_2, EtOAc, and MeOH (each 5 × 1 L, 24 h), and the four individual solvent extracts were concentrated under vacuum. When the residues were bioassayed against the test organisms, only the EtOAc (2.1 g) and MeOH (20.3 g) extracts were active. The EtOAc extract was fractionated on Si gel by VLC using a CH_2Cl_2−MeOH gradient (95:5→0:100) and followed by TLC. Of the five fractions obtained, only one was active. This one was further purified by VLC on Si gel, eluting with CH_2Cl_2−MeOH (90:10→60:40). Recrystallization from

MeOH−*i*-Pr_2O gave **1** (210 mg). The residue from the MeOH extract was taken up in MeOH (100 mL) and poured into Et_2O (1 L) with rapid stirring. After filtering off the precipitate, the filtrate, which contained the activity, was concentrated and fractionated by VLC on Si gel, eluting with CH_2Cl_2−MeOH (90:10→0:100). The active fraction was subjected to two further purification steps by VLC on Si gel, the first using CH_2Cl_2−MeOH (90:10→50:50) for the eluting gradient and the second using EtOAc−MeOH (90:10→70:30). The active fraction was recrystallized from MeOH−*i*Pr_2O to give **1** (930 mg). The total yield of **1** from both the EtOAc and MeOH extracts represented 0.5% of the dry weight of the fruit.

4-Hydroxyphenyl 6-*O*-[(3*R*)-3,4-dihydroxy-2-methylenebutanoyl]-*β*-D-glucopyranoside (1): colorless crystals (EtOH−*i*Pr_2O) (753 mg); mp 163−165 °C; $[\alpha]^{20}_D$ −60.3° (*c* 2.5, MeOH); found, C 52.37%; H 5.54%, calcd for $C_{17}H_{22}O_{10}$, C 52.85%, H, 5.74%; UV(MeOH) λ_{max} (log ϵ) 286 (3.43) nm; IR (KBr disk) υ_{max} 3521 (OH), 3390 (br, OH), 1704 (C=O), 1629, 1516, 1468, 1457, 1374, 1353, 1284, 1253, 1223, 1077, 1052, 1017, 835, 778, 624 cm^{-1}; ^1H NMR (CD_3COCD_3, 500 MHz) δ 6.93 (2H, d, J = 8.9 Hz, H-3″, H-5″), 6.69 (2H, d, J = 8.9 Hz, H-2″, H-6″), 6.38 (1H, d, J = 1.5 Hz, H-5a′), 6.03 (1H, d, J = 1.5 Hz, H-5b′), 4.81 (1H, d, J = 8.0 Hz, H-1), 4.61 (1H, ddt, J = 1.5, 4.0, 6.5 Hz, H-3′), 4.55 (1H, dd, J = 2.5, 12.0 Hz, H-6a), 4.28 (1H, dd, J = 7.0, 12.0 Hz, H-6b), 3.75 (1H, ddd, J = 2.5, 7.0, 10.0 Hz, H-5), 3.72 (1H, dd, J = 4.0, 11.5 Hz, H-4a′), 3.57 (1H, t, J = 9.0 Hz, H-3), 3.49 (1H, dd, J = 9.0, 10.0 Hz, H-4), 3.47 (1H, dd, J = 8.0, 9.0 Hz, H-2), 3.44 (1H, dd, J = 7.0, 11.5 Hz, H-4b′); ^{13}C NMR (CD_3OD, 75.5 MHz) δ 167.5 (s, C-1′), 154.1 (s, C-4″), 152.4 (s, C-1″), 142.3 (s, C-2′), 127.2 (t, C-5′), 119.8 (d, C-3″, C-5″), 116.9 (d, C-2″, C-6″), 103.7 (s, C-1), 78.0 (d, C-3), 75.5 (d, C-5), 75.1 (d, C-2), 72.3 (d, C-3′), 72.0 (d, C-4), 66.9 (t, C-4′), 65.4 (t, C-6); FABMS (negative, glycerol) m/z $[M - H]^-$ 385 (56), 355 (46), 271 (100); FABMS (positive, NBA) m/z $[MNa]^+$409, $[MH]^+$387.

Acid Hydrolysis of 1. Compound **1** (340 mg, 0.9 mM) was added to 0.1 M HCl (10 mL) and stirred at 80 °C until TLC showed the disappearance of the starting material. After cooling, the solution was saturated with NaCl and extracted with EtOAc (5 × 5 mL). The organic extract was purified by VLC on Si gel (EtOAc−hexane; 50:50→100:0) giving hydroquinone, D-glucose (both identified by comparison with authentic samples), and (*R*)-3-hydroxy-2-methylene-*γ*-butyrolactone [(+)-tulipalin B] (**2**) as a colorless oil (91 mg): $[\alpha]^{20}_D$ +85.5°-(*c* 5.1, MeOH); lit.[6] $[\alpha]^{25}_D$ +83°; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.33 (1H, d, J = 2.0 Hz, H-5a), 6.00 (1H, d, J = 2.0 Hz, H-5b), 4.91 (1H, m, H-3), 4.52 (1H, dd, J = 6.6, 10.1 Hz, H-4a), 4.20 (1H, dd, J = 3.6, 10.1 Hz, H-4b); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ 170.1 (s, C-1), 137.4 (s, C-2), 126.9 (t, C-5), 73.6 (t, C-4), 67.1 (d, C-3); EIMS (70 eV) m/z $[MH]^+$ (self-protonation) 115 (17), $[M]^+$ 114 (2), 84 (92), 56 (100), 55 (92); HREIMS (70 eV) m/z 114 $[M]^+$, found 114.0320; $C_5H_6O_3$ requires 114.0317.

Single-Crystal X-ray Analysis of 1. Crystal data: $C_{17}H_{22}O_{10}$, monoclinic space group $P2_1$, a = 12.121(2) Å, b = 5.090(1) Å, c = 31.316(4) Å, β = 90.61(1)°, V = 1932.1(5) $Å^3$, Z = 4, D_{calcd} =1.328 g cm^{-3}, $F(000)$ = 816. Intensity data were collected on a Rigaku AFC6R diffractometer using monochromated Cu Kα radiation from a rotating anode X-ray generator. The structure can be regarded as a $P2_12_12_1$ structure modified to $P12_11$ with the change of β to 90.61(1)°. As refinement of the structure is of considerable crystallographic interest, full details will be published in a crystallographic journal (Rae, A. D.; Willis, A. C.; MacLeod, J. K.; Rasmussen H. B. *Z. Krist.*, manuscript in preparation). Figure 1 shows a thermal ellipsoid diagram of a molecule of **1**.

Acknowledgment. We wish to thank Lyn Meredith, National Botanic Gardens, Canberra, for his assistance with the plant collection and Margot Anderson for her help with the bioassays. We also thank Nigel Perry for communicating his results on compound **1** to us prior to publication. H.B.R. thanks the Australian Government for the award of an Overseas Postgraduate Scholarship.

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NP970006A