Antimicrobial and Cytotoxic Phenolic Glycoside Esters from the New Zealand Tree *Toronia toru*

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Two new compounds, 4-hydroxyphenyl 6-*O*-(4-hydroxy-2-methylenebutanoyl)-*β*-D-glucopyranoside (**1**) and 4-hydroxyphenyl 6-*O*-[(3*R*)-3,4-dihydroxy-2-methylenebutanoyl]-*β*-D-glucopyranoside (**2**), have been isolated from foliage of *Toronia toru*. Compound **2** was the main antimicrobial component (7 mg/g of dry foliage) of the crude extract and also showed significant cytotoxic activity against P-388 leukemia cells.

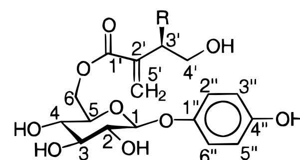
We have been screening New Zealand plants for bioactive natural products with the aim of developing new pharmaceuticals and agrochemicals.[1] An extract from *Toronia toru* (Cunn.) L. Johnson et B. Briggs (Maori name "toru") showed strong cytotoxic and antimicrobial activity. Similar bioactivity was noted in an earlier screening program,[2] but no Maori traditional medicinal uses have been recorded for this plant.[3,4] *T. toru* is a small tree native to the north of the North Island of New Zealand.[5] It was previously known as *Persoonia toru* A. Cunn.[6] but was recently assigned as the only species in the genus *Toronia*, of the family Proteaceae.[7]

We could not find any references to previous chemical studies on *T. toru*, but in view of the compounds identified in the present investigation, it is noteworthy that arbutin (**3**) and hydroquinone have been detected in several species of Proteaceae.[8] We now report the isolation and structure identification of two new phenolic glycoside esters **1** and **2**, which are the main antimicrobial and cytotoxic components from *T. toru*. During the course of this work, compound **2** was independently found to be the main antimicrobial component in the fruits of an Australian tree *Persoonia linearis* × *pinifolia*.[9]

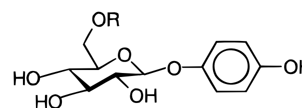
A crude extract of *T. toru* leaf and stem showed broad-spectrum antibacterial activity against both Gram-positive bacteria (*Bacillus subtilis, Dermatophilus congolensis*, and *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), and against the dermatophyte *Trichophyton mentagrophytes*. The *T. toru* extract also showed cytotoxic activity against both BSC monkey kidney cells and P-388 leukemia cells (P-388; IC_{50} ca. 8 μg/mL).

Assays against *B. subtilis* were used to guide the isolation of the main antibacterial component(s). Reversed-phase (C_{18}) flash chromatography gave most of the antibacterial activity in the highest polarity fractions, eluted with H_2O. Further reversed-phase chromatography was carried out on an ethyl-functionalized stationary phase, as this was expected to be more retentive of polar compounds. Two related compounds, **1** and **2**, were obtained pure from this column. Compound **2** was the main antibacterial component in the extract.

The NMR spectra of compounds **1** and **2** (Table 1) both showed ^{1}H signals at 4.96 ppm (d, 7 Hz) and ^{13}C signals at 103.6 and 103.7 ppm (d, $^1J_{C-H}$ = 164 Hz), suggestive of *β*-glucopyranosides. These spectra also contained signals, at 6.98 and 6.99 ppm (2H, d, 9 Hz) and 6.82 and 6.84 ppm (2H, d, 9 Hz), as found in hydroquinone derivatives. The UV spectra of compounds **1** and **2** were similar to the UV spectrum of pyroside (**4**). Therefore, it seemed possible that these compounds from *T. toru* were derivatives of arbutin (**3**). This hypothesis was supported by close matches in the ^{1}H- and ^{13}C-NMR data of **1** and **2** with those of pyroside (**4**).[10]



1 R=H
2 R=OH



3 R=H
4 R=Ac

The ^{1}H- and ^{13}C-NMR spectra of compound **1** showed the following groups additional to the arbutin skeleton: ester C=O, C=CH$_2$, C−CH$_2$−C, and C−CH$_2$−O (Table 1). These required a molecular formula of $C_{17}H_{22}O_9$, which was supported by HRFABMS. The full structure of **1** was shown by a two- and three-bond ^{1}H−^{13}C correlation NMR experiment (HMBC, Table 1). This showed the presence of a 2-methylene-4-hydroxybutanoic acid unit, as well as the expected *β*-glucopyranoside and hydroquinone units. The linkages between these units were established by correlations from both glucose H-6′s to the ester carbonyl C-1′, and from the glucose H-1 to one aromatic signal, C-1″. Therefore, this compound has the previously unreported structure, **1**. A few other glycosides esterified at C-6 with 2-methylene-4-hydroxybutanoic acid are known,[11,12] with the simplest analogue of **1** being 6-tuliposide A (**5**).[12] The

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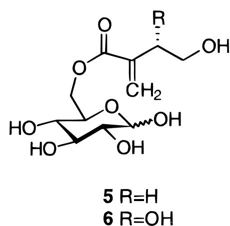
Table 1. ^{1}H- and ^{13}C-NMR Data of Compounds **1** and **2**[a]

position	1			2	
	^{1}H[b]	^{13}C	HMBC, ^{1}H to ^{13}C correlations	^{1}H	^{13}C
1	4.96 (d, 7)	103.6 (br d, 164)	152.8	4.96 (d, 7)	103.7
2	3.59 (dd, 7, 9)	75.5 (dd, 147, 6)	103.6, 78.2	3.5−3.7	75.6
3	3.63 (t, 9)	78.2 (br d, 148)	75.5	3.5−3.7	78.1
4	3.53 (t, 9)	72.7 (br d, 144)	78.2, 66.4	3.5−3.7	72.6
5	3.80 (ddd, 2, 7, 9)	76.2 (br d, 148)	72.7	3.82 (ddd, 2, 7, 9)	76.2
6	4.56 (dd, 2, 12)	66.4 (t, 151)	171.0, 72.7	4.54 (dd, 2, 12)	66.4
6	4.34 (dd, 7, 12)		171.0, 76.2	4.40 (dd, 7, 12)	
1′		171.0 (s)			169.5
2′		138.8 (s)			141.5
3′	2.52 (t, 7)	36.7 (br t, 131)	171.0, 138.8, 131.6, 62.6	4.61 (dd, 6, 3)	73.0
4′	3.67 (t, 7)	62.6 (tt, 144, 5)	138.8, 36.7	3.5−3.7	67.0
5′	5.78 (br s)	131.6 (tt, 161, 6)	171.0, 36.7	6.04 (br s)	130.9
5′	6.27 (s)		171.0, 138.8, 36.6	6.42 (s)	
1″		152.8 (s)			152.8
2″	6.98 (d, 9)	121.0 (dd, 161, 5)	153.9, 152.8, 121.0, 118.8	6.99 (d, 9)	121.1
3″	6.82 (d,9)	118.8 (dd,161,5)	152.8,118.8	6.84 (d,9)	118.8
4″		153.9 (s)			153.9
5″	6.82 (d,9)	118.8 (dd,161,5)	152.8,118.8	6.84 (d,9)	118.8
6″	6.98 (d,9)	121.0 (dd,161,5)	153.9,152.8,121.0,118.8	6.99 (d,9)	121.1

[a] In D_2O, δ in ppm (multiplicity, J in Hz). [b] One bond ^{1}H−^{13}C correlations confirmed by HMQC.

^{1}H- and ^{13}C-NMR data reported for the β-anomer of **5** match our data for the ester unit of **1** very well.[13]

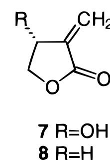


5 R=H
6 R=OH

The HRFABMS of compound **2** showed the presence of one further oxygen atom compared to **1**. The ^{1}H- and ^{13}C-NMR spectra of **2** showed very similar signals to the hydroquinone and β-glucopyranoside signals of **1** (Table 1). The NMR spectra of **2** did not show the CH_2 -3′ signals of **1**, but did show an additional −CHO− group [^{1}H 4.61 ppm (dd, 6, 3 Hz); ^{13}C 73.0 ppm]. Therefore, this compound was the 3,4-dihydroxy 2-methylbutanoate ester **2**. 1-Tuliposide B (**6**) has the same ester group,[12] but structure **2** has not previously been reported in the literature. MacLeod *et al.* have also isolated compound **2** from a hybrid between *P. pinifolia* and *P. linearis*.[9] They determined the relative stereochemistry of **2** by X-ray crystallography, and the absolute stereochemistry of CHOH-3′ by hydrolysis of **2** to lactone **7**.[9] Compound **2** from *T. toru* had the same ^{1}H- and ^{13}C-NMR spectra as the compound from *P. linearis* × *pinifolia* and a similar optical rotation.

Compounds **1** and **2** were major components of our bulk extract of *T. toru* foliage, with isolated yields of 7 mg/g dry foliage for **2** and 3 mg/g for **1**. Characteristic signals of **1** and **2** were prominent in the ^{1}H-NMR spectrum of the crude extract. We used biological assays and ^{1}H-NMR spectra to check whether **1** and **2** were present in other collections of *T. toru*. Five different *T. toru* samples all showed the same strong antimicrobial and cytotoxic activity as our original collection, and all showed characteristic ^{1}H-NMR peaks of **1** and **2**. Therefore, these results suggest that these novel phenolic glycoside esters are characteristic of *T. toru* foliage. It would be interesting to know whether they also occur widely in the closely related genus *Persoonia*. MacLeod *et al.* only reported **2** in their extract of *P. linearis* × *pinifolia* fruits.[9]

The antimicrobial and cytotoxic activities of new compounds **1** and **2** are summarized in Table 2. Compound **2** showed broad-spectrum antibacterial activity, plus antifungal activity against *T. mentagrophytes*, and cytotoxic activity against both P-388 leukemia and BSC monkey kidney cells. Compound **1** was only active (at the doses tested) against the BSC cells. The antimicrobial and cytotoxic activity of compound **2** could be due to formation of lactone **7**, which is both antimicrobial and cytotoxic.[12,14] α-Methylene lactone **7** is probably toxic because of Michael addition to biological nucleophiles. The corresponding lactone **8**, which might be released by hydrolysis of **1**, was antimicrobial and cytotoxic in our assays (Table 2). The differing biological activities of **1** and **2** could be due to different rates of lactone formation in the assay media or within the target cells. Hydroquinone, another possible product from hydrolysis of **1** and **2**, was also antimicrobial and cytotoxic (Table 2), although arbutin (**3**) was inactive. These phenolic glycoside esters (**1** and **2)** may represent another class of "post-inhibitin" in plants: inactive glycosides from which active toxins are released by enzymic hydrolysis, following microbial invasion or herbivore attack on foliage.[15]



7 R=OH
8 R=H

Experimental Section

General Experimental Procedures. All solvents were distilled before use and were removed by rotary evaporation at temperatures up to 45 °C. Octadecyl-functionalized Si gel (Aldrich) was used for C_{18} chromatography, and ethyl-functionalized Si gel (International Sorbent Technology) was used for C_2 chromatography. TLC was carried out using Merck DC-Plastikfolien Kieselgel 60 F_{254}. MS, IR, and UV spectra were recorded on Kratos MS80, Perkin-Elmer 1600, and JASCO 7800 UV−vis spectrometers, respectively. Optical rotations were recorded on a JASCO DIP-1000 polarimeter. NMR spectra, in D_2O solution at 25 °C,

Table 2. Biological Assay Results for Compounds **1** and **2** and Related Compounds

compound	dose (μg/disk)	antimicrobial activity[a]				cytotoxicity	
		B. subtilis	*E. coli*	*P. aeruginosa*	*T. mentagrophytes*	BSC[b]	P-388[c]
1	120	0	0	0	0	100	>25
	60	0	0	0	0	100	
	30	0	0	0	0	100	
	15	NT	NT	NT	NT	50	
2	120	5	4	3	5	100	3
	60	1	2	1	0	100	
	30	0	0	0	0	50	
3	120	0	0	0	0	50	>25
hydroquinone	120	9	5	3	10	100	3
	60	4	0	3	0	100	
	30	3	0	2	0	75	
tulipalin A (**8**)	120	10	5	4	8	100	0.8
	60	0	0	0	0	100	
	30	0	0	0	0	100	
	15	0	0	0	0	100	

[a] Width of zone of growth inhibition (mm), NT = not tested. [b] % of well showing cytotoxic effects. [c] IC_{50} (μg/mL).

were recorded at 300 MHz for ^1H and 75 MHz for ^{13}C on a Varian VXR-300 spectrometer. Chemical shifts were referenced to the main signal of 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (DSS, Aldrich) at 0 ppm. Arbutin (Sigma), lactone **8** (Aldrich), and hydroquinone were commercial samples.

Collection and Screening. Foliage (leaves and stems) was collected from a tree in the Dunedin Botanical Gardens in September 1995. A voucher specimen, 950919-01, has been kept in the Plant Extracts Research Unit Collection. Further samples of *T. toru* foliage were obtained in August 1996, from the Auckland Regional Botanic Gardens (voucher 960807-01) and from Otari Native Botanical Gardens, Wellington (plants from four different source locations, vouchers 960820-01 to -04). Foliage was air-dried at 35 °C for 3 days. Initial screening was carried out using an extract produced by shaking milled material (5.0 g) overnight in EtOH (95% EtOH−5% H_2O; 50 mL). A subsample (1 mL) of this extract was dried, dissolved in D_2O (1 mL), and filtered prior to ^1H-NMR analysis (200 MHz, Varian Gemini). Spectra, referenced to **H**OD at 4.70 ppm, were examined for characteristic peaks of **1** at 6.15 ppm (s), 5.65 ppm (s), and 2.30 ppm (t), and of **2** at 6.30 ppm (s) and 5.90 ppm (s).

Extraction and Isolation. A bulk extract of dried plant material (950919-01, 103 g) was prepared by blending with EtOH (1 × 600 mL and 2 × 450 mL). The solvent was removed from the combined, filtered extracts to give a green gum (20.9 g, inhibition zone against *B. subtilis* 2 mm, 120 μg/disk; abbreviated as Bs 2 @120). A subsample of this extract (6 g) was precoated onto 10 g of C_{18} Si gel and loaded onto a 50-g C_{18} Si gel column, preconditioned with H_2O. The column was developed in 37-mL steps with H_2O through to MeCN, finishing with 1:1 MeCN−$CHCl_3$. The most active fractions were eluted with H_2O (1.62 g, Bs 2 @ 120 μg/disk). A subsample (1 g) of these active fractions was precoated onto 2 g of C_2 Si gel and loaded onto a 20-g C_2 Si gel column, preconditioned with H_2O. This column was developed in 20-mL steps with H_2O and combined on the basis of TLC on Si gel. The most potent antimicrobial component, from combined fractions 4−8 (Bs 6 @ 120 μg/disk) was pure **2** (130 mg). A later sample, from combined fractions 13−15 (Bs 0 @ 120 μg/disk) contained pure **1** (62 mg).

4-Hydroxyphenyl 6-*O*-(4-hydroxy-2-methylene-butanoyl)-β-D-glucopyranoside (1): pale orange gum; $[\alpha]^{19}_D$ +18° (*c* 2.5, MeOH); UV (EtOH) λ_{max} (log ε) 285 (3.61) nm; IR (Nujol) ν_{max} 3274 (br), 1708, 1634, 1511 cm^{-1}; ^1H- and ^{13}C-NMR data in Table 1; FABMS (glycerol + NaCl) *m/z* 393.1149 (M + Na^+, $C_{17}H_{22}O_9$-Na requires 393.1161); Si gel TLC (100:13.5:10 EtOAc−MeOH−H_2O) R_f 0.3, UV-active plus blue with Berlin Blue reagent.

4-Hydroxyphenyl 6-*O*-[(3*R*)-3,4-dihydroxy-2-methylenebutanoyl]-β-D-glucopyranoside (2): colorless gum; $[\alpha]^{19}_D$ −48° (*c* 2.5, MeOH); UV (EtOH) λ_{max} (log ε) 285 (3.89) nm; IR (Nujol) ν_{max} 3305 (br), 1710 (br), 1634 (br) cm^{-1}; ^1H- and ^{13}C-NMR data in Table 1, ^1H-NMR (in CD_3COCD_3) and ^{13}C-NMR (in CD_3OD) spectra match MacLeod *et al.*;[9] FABMS (glycerol) *m/z* 387.1283 (MH^+ $C_{17}H_{23}O_{10}$ requires 387.1291); Si gel TLC (100:13.5:10 EtOAc−MeOH−H_2O) R_f 0.3, UV-active plus blue with Berlin Blue reagent.

Biological Testing. Details of antimicrobial and cytotoxicity assays have been given previously.[16]

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