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Tyrosine kinase inhibitors from the rainforest tree *Polyscias murrayi*

Malcolm S. Buchanan, Anthony R. Carroll, Annette Edser, John Parisot, Rama Addepalli, Ronald J. Quinn *

Natural Product Discovery, Eskitis Institute, Griffith University, Brisbane, Qld. 4111, Australia

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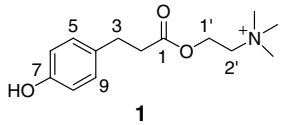
Abstract

A series of 3-(4-hydroxyphenyl) propanoic acid derivatives, which inhibit Itk (interleukin-2 inducible T-cell kinase), a Th2-cell target, were isolated from the Australian rainforest tree *Polyscias murrayi*. The new compound 3-(4-hydroxyphenyl) propionyl choline and a 2:1 mixture of the new compounds 3,4–di-*O*-3-(4-hydroxyphenyl) propionyl-1,5-dihydroxycyclohexanecarboxylic acid and 3,5–di-*O*-3-(4-hydroxyphenyl) propionyl-1,4-dihydroxycyclohexanecarboxylic acid were isolated along with two known compounds 3-(4-hydroxyphenyl) propanoic acid and 3-(3,4-hydroxyphenyl) propanoic acid. Their structures were determined by 1D and 2D NMR spectroscopy. The assay results suggest that both the 3-(4-hydroxyphenyl) propanoate and carboxyl moieties contribute to Itk activity of the compounds.

Keywords: *Polyscias murrayi*; Araliaceae; Tyrosine kinase inhibitors; Interleukin-2 inducible T-cell kinase; Natural products

1. Introduction

Interleukin-2 inducible T-cell kinase (Itk) is a non-receptor tyrosine kinase expressed mainly on $CD4^+$ T-cells (Siliciano et al., 1992; Lin et al., 2004). Selective inhibitors of Itk have use as immunosuppressive agents in the treatment of asthma, rheumatoid arthritis, and other immunological and inflammatory disorders. In a natural product screening campaign to find inhibitors of Itk, a Th2-cell target, the flowers of *Polyscias murrayi* (F. Muell.) Harms (Araliaceae) were studied. *P. murrayi* is widespread in the rainforest in Eastern Australia and forms a large canopy tree up to 30 m tall. The 3-(4-hydroxyphenyl) propanoic acid derivatives **1** and a 2:1 mixture of compounds **2a** and **2b**, were isolated along with two known compounds 3-(4-hydroxyphenyl) propanoic acid (**3**) and 3-(3,4-dihydroxyphenyl) propanoic acid (**4**). Quinic acid derivatives similar to **2a** and **2b** have been isolated previously (Wang et al., 2003; Um et al., 2002; Hiroyuki et al., 2000; Scholz et al., 1994; Cheminat et al., 1998; Hur et al., 2004). Moreover, (−)-3,5-dicaffeoyl-*muco*-quinic acid (**5**) has been reported to increase tyrosine kinase activity (Hur et al., 2004). The isolation and structure elucidation of these compounds are discussed, together with their Itk inhibitory activity.



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* Corresponding author. Tel.: +61 7 3875 6000; fax: +61 7 3875 6001.
E-mail address: r.quinn@griffith.edu.au (R.J. Quinn).

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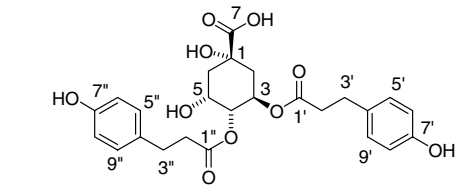
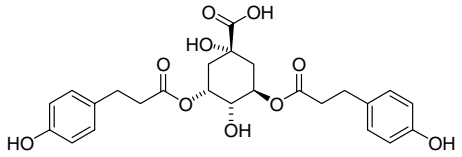
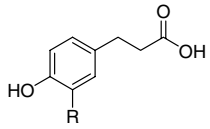


2a



2b



3 R = H

4 R = OH

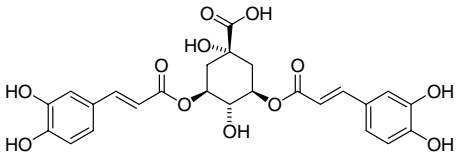


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Table 1
^{1}H (600 MHz), ^{13}C (150 MHz) and gHMBC NMR data for compound **1**[a,b]

Position	^{13}C	^{1}H	HMBC
1	173.8 *s*	–	–
2	35.3 *t*	2.61 (*t*, *J* = 7.5 Hz), 2H	1, 3
3	29.2 *t*	2.76 (*t*, *J* = 7.5 Hz), 2H	1, 2, 5, 9
4	118.7 *s*	–	–
5	129.0 *d*	7.01 (*d*, *J* = 8.5 Hz)	7, 9
6	115.1 *d*	6.67 (*d*, *J* = 8.5 Hz)	5, 7, 8
7	155.6 *s*	–	–
8	115.1 *d*	6.67 (*d*, *J* = 8.5 Hz)	6, 7, 9
9	129.0 *d*	7.01 (*d*, *J* = 8.5 Hz)	5, 7
1′	57.7 *t*	4.43 (*m*), 2H	1
2′	63.7 *t*	3.62 (*m*), 2H	1′, N–Me
N–(Me)$_3$	52.9 *q*	3.08 (*s*)	2′, N–Me
7-OH	–	9.21 (brs)	–

[a] Assignments confirmed by 2D experiments (gCOSY, ROESY, gHSQC and gHMBC).
[b] Multiplicities were determined by DEPT experiments.

2. Results and discussion

The ground flower sample of *P. murrayi* was extracted and fractionated with MeOH/H_2O and chromatographed through MPLC columns of polyamide gel and reversed-phase C18. Final purification by reversed-phase C18 HPLC yielded the new compounds **1**, **2a**, and **2b**. The compounds 3-(4-hydroxyphenyl) propanoic acid (**3**) (Pouchert and Behnke, 1993a) and 3-(3,4-dihydroxyphenyl) propanoic acid (**4**) (Pouchert and Behnke, 1993b) were also isolated and their structures determined from MS and NMR data, together with previously published data.

The molecular formula of **1** was determined to be $[C_{14}H_{22}NO_3]^+$ by HRESIMS (*m*/*z* 252.1597, calculated 252.1594). The ^{1}H NMR spectrum (Table 1) of compound **1** contains signals for a *para*-disubstituted benzene ring {δ_H 7.01 (*d*, *J* = 8.5 Hz), 6.67 (*d*, *J* = 8.5 Hz)} and two methylenes (δ_H 2.76, 2.61). These signals, together with a phenolic proton (δ_H 9.21) and an ester carbonyl (δ_C 173.8) and their mutual two-dimensional (2D) correlations, revealed a 3-(4-hydroxyphenyl) propanoate moiety. In total, 10 carbon signals were identified in the ^{13}C NMR spectrum. The nine-proton singlet at δ_H 3.08 correlated to a carbon at δ_C 52.9 in both the gHSQC and gHMBC spectra. This suggested the presence of a trimethylammonium moiety. The remaining signals in the ^{1}H NMR spectrum were two methylene groups at δ_H 3.62 and 4.43, which correlated together in gCOSY, the former of which had a gHMBC correlation to the trimethylammonium (δ_C 52.9). The above information indicated that compound **1** was 3-(4-hydroxyphenyl) propionyl choline.

Compounds **2a** and **2b** were isolated together as a 2:1 mixture of isomers, based on ^{1}H and ^{13}C NMR data. The (−)-ESIMS shows an $[M-H]^-$ signal at *m*/*z* 487 from which a molecular formula of $C_{25}H_{28}O_{10}$ was assigned. Looking at the data for the main component (**2a**) of the mixture the ^{1}H NMR and ^{13}C NMR spectra (Table 2) revealed the presence of two 3-(4-hydroxyphenyl) propanoate groups. An oxygenated cyclohexane ring was indicated, by the successive gCOSY correlations between δ_H 2.04 (H-2β), 5.12 (H-3), 4.79 (H-4), 4.04 (H-5) and 2.10/1.79 (H-6β/H-6α), with an oxygenated quaternary carbon completing the ring {δ_C 72.3 (C-1)}. This was supported by data from the gHMBC, which furthermore indicated the attachment sites of the 3-(4-hydroxyphenyl) propanoate groups on the six-membered ring. Thus, the correlations between H-3 (δ_H 5.12) and C-1′ (δ_C 171.0) and between H-4 (δ_H 4.79) and C-1″ (δ_C 171.5) revealed the attachment sites of the two ester groups. To correspond with the molecular formula there remained two hydroxyls and a carboxyl. Their attachment sites on the six-membered ring were obvious, compound **2a** being made up of two 3-(4-hydroxyphenyl) propanoate residues and a 1,3,4,5-tetrahydroxycyclohexanecarboxylic acid residue. The relative stereochemistry was established from chemical shifts, *J*-coupling (Table 2) and ROESY data. Hence,

Table 2
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for isomers **2a** and **2b**[a,b]

Position	**2a**		**2b**	
	^{13}C	^{1}H	^{13}C	^{1}H
1	72.3 *s*	–	72.8 *s*	–
2	36.5 *t*	α 1.86 (*m*), β 2.04 (*m*)	34.4 *t*	α 1.88 (*m*), β 2.04 (*m*)
3	67.6 *d*	5.12 (*td*, *J* = 8.0, 4.2 Hz)	70.9 *d*	4.99 (*m*)
4	73.2 *d*	4.79 (*dd*, *J* = 8.0, 2.5 Hz)	67.6 *d*	3.70 (*m*)
5	65.8 *d*	4.04 (*m*)	70.5 *d*	5.05 (*dt*, *J* = 7.6, 3.8 Hz)
6	37.4 *t*	α 1.79 (*m*), β 2.10 (*m*)	35.7 *t*	α 1.85 (*m*), β 2.02 (*m*)
7	174.3 *s*	–	175.0 *s*	–
–COOH	–	12.42 (brs)	–	12.42 (brs)
4-OH/5-OH	–	n.o.	–	5.17 (*d*, *J* = 4.8 Hz)
1′	171.0 *s*	–	171.1 *s*	–
2′	35.6[c] *t*	a 2.54 (*m*), b 2.45 (*m*)	35.8[c] *t*	a 2.54 (*m*), b 2.45 (*m*)
3′	29.3[d] *t*	a 2.73 (*m*), b 2.68 (*m*)	29.3 *t*	a 2.73 (*m*), b 2.68 (*m*)
4′	130.4[e] *s*	–	130.4[d] *s*	–
5′	128.9 *d*	7.00 (*m*)	129.0 *d*	7.00 (*m*)
6′	115.0 *d*	6.65 (*m*)	115.0 *d*	6.65 (*m*)
7′	155.4 *s*	–	155.4[e] *s*	–
8′	115.0 *d*	6.65 (*m*)	115.0. *d*	6.65 (*m*)
9′	128.9 *d*	7.00 (*m*)	129.0 *d*	7.00 (*m*)
7′-OH	–	9.13 (brs)	–	9.13 (brs)
1″	171.5 *s*	–	171.6 *s*	–
2″	35.5[c] *t*	a 2.54 (*m*), b 2.45 (*m*)	35.7[c] *t*	a 2.54 (*m*), b 2.45 (*m*)
3″	29.4[d] *t*	a 2.73 (*m*), b 2.68 (*m*)	29.3 *t*	a 2.73 (*m*), b 2.68 (*m*)
4″	130.3[e] *s*	–	130.7[d] *s*	–
5″	128.9 *d*	7.00 (*m*)	129.0 *d*	7.00 (*m*)
6″	115.0 *d*	6.65 (*m*)	115.0 *d*	6.65 (*m*)
7″	155.4 *s*	–	155.3[e] *s*	–
8″	115.0. *d*	6.65 (*m*)	115.0 *d*	6.65 (*m*)
9″	128.9 *d*	7.00 (*m*)	129.0 *d*	7.00 (*m*)
7″-OH	–	9.13 (brs)	–	9.13 (brs)

n.o., Not observed.
[a] Assignments confirmed by 2D experiments (gCOSY, ROESY, gHSQC and gHMBC).
[b] Multiplicities were determined by gHSQC experiments.
[c–e] Signals within the same column are interchangeable.

there were ROESY correlations between H-4 and H-5, and between H-5 and H-6α. The two 3-(4-hydroxyphenyl) propanoate groups were equatorially orientated on the cyclohexane ring. The downfield shift of the axial protons H-2β and H-6β relative to H-2α and H-6α seems to indicate that they were being deshielded by the carboxyl at C-1. However, the equatorial nature of the carboxyl was only tentatively assigned as the 3-(4-hydroxyphenyl) propanoate groups may have anisotropic effects as well. Compound **2a** was therefore identified as 3,4–di-*O*-3-(4-hydroxyphenyl) propionyl-1,5-dihydroxycyclohexanecarboxylic acid.

The minor isomer **2b** differs from **2a** only in the attachment of groups to the cyclohexane ring. From the ^{1}H and ^{13}C NMR data (Table 2) and supported by 2D NMR information it is clear that in **2b**, the 3-(4-hydroxyphenyl) propanoate residue attached at C-4 in **2a** was attached to C-5 in **2b**. There were gHMBC correlations between H-3 (δ_H 4.99) and C-1′ (δ_C 171.1 and between) H-5 (δ_H 5.05) and C-1″ (δ_C 171.6) indicating the ester attachment sites. Similarly to **2a**, the relative stereochemistry for **2b** was also established from chemical shifts, *J*-coupling (Table 2) and ROESY data. Thus, there were ROESY correlations between H-4 and H-5, and between H-5 and H-6β. One 3-(4-hydroxyphenyl) propanoate group was equatorially orientated on the cyclohexane ring (C-5) while the other was axial (C-3). Compound **2b** was therefore identified as 3,5–di-*O*-3-(4-hydroxyphenyl) propionyl-1,4-dihydroxycyclohexanecarboxylic acid.

The inhibitory activity of the non-receptor tyrosine kinase, Itk, for compounds **1–4** is tabulated in Table 3. The potency of compounds **2a/2b** and **3** are similar, while compounds **1** and **4** are about eight times less potent compared with **2a/2b** and **3**. This suggests that both

Table 3
Itk inhibition of compounds **1–4**

Compound	IC_{50} (μm)
1	508
2a/2b	58
3	64
4	418

the 3-(4-hydroxyphenyl) propanoate and carboxyl moieties contribute to the activity of compounds **1–4**. Further assays would have needed to be run to determine whether these natural products are selective inhibitors of Itk.

3. Experimental section

3.1. General experimental procedures

Water was Millipore Milli-Q PF filtered, while all other solvents used were Omnisolv HPLC grade. A Hypersil BDS C18 5 μm (10 mm × 250 mm i.d.) was used for semi-preparative HPLC. A Waters 600 pump fitted with a 996 Photodiode Array Detector and 717 plus Autosampler was used for the semi-preparative separations. NMR spectra were recorded at 30 °C on a Varian Inova 600 MHz NMR spectrometer. Samples were dissolved in DMSO-d_6 (^{1}H δ 2.50 and ^{13}C δ 39.5 ppm). HRESIMS was measured on a Bruker BioAPEX 47e mass spectrometer equipped with a Bradford CT 06405 electrospray ion source. Europium-labelled anti-phosphotyrosine[66] antibodies (PT66(Eu)) and highly fluorescent streptavidin-conjugated allophycocyanin (S-APC) were purchased from Perkin Elmer Life Sciences-Wallac (Turku, Finland). Purified (85–90%) GST-Itk (290 μg/ml; batch 7) enzyme in sample buffer (50 mM Tris/HCl, 150 mM NaCl, 5% (w/v) Mannitol, 1 mM DTT and 30% (v/v) glycerol) was obtained from AstraZeneca-ABL (Södertälje). The biotinylated substrate, AR-D101035, was obtained from AstraZeneca-Lund. Chemical grade reagents: glycerol, Hepes, $MgCl_2$, Brij 35, Tris/HCl, EDTA, DTT, NaCl, BSA, DMSO, ATP and PVP were obtained from Sigma–Aldrich (Sydney, Australia). The reference inhibitor compound, damnacanthal was purchased from Calbiochem (Sydney, Australia). Solid black, 384-well microtitre plates were purchased from Greiner Labortechnik (Frickenhausen, Germany).

3.2. Plant material

Polyscias murrayi (F.Muell.) Harms [Kingdom-Plantae, Phylum-Spermatophyta, Class-Angiospermae, Family-Araliaceae] was collected on the Lamington Plateau, Queensland, Australia (28°12′S, 153°05′E) on January 12th, 1994. A voucher sample, 11201940200000350, has been deposited at the Queensland Herbarium, Brisbane, Queensland, Australia.

3.3. Assay principle

The assay measures the inhibition of the phosphorylation of biotinylated peptide substrate by the Itk enzyme measured as a decrease in time resolved fluorescence resonance energy transfer (TR-FRET). This is using europium tagged anti-phosphotyrosine[66] antibodies (PT66Eu) as a donor of energy to the streptavidin-labelled allophycocyanin (S-APC) fluorescent acceptor molecules. The measurement was expressed as a ratio of the time resolved fluorescence counts determined at a wavelength of 665 nm (acceptor) and 625 nm (donor).

3.4. Assay procedure

Screening of compounds **1–4** was performed at 25 μg/well extract concentration. Extract (1 μl) plus 9ul water were aspirated and dispensed into solid black 384-well plates using a CCS-Packard MiniTrac liquid handling device, and the plates stored at 4 °C. The following day, stock Itk (290 μg/ml) enzyme kept on ice was diluted with assay buffer (50 mM Hepes, 10 mM $MgCl_2$, 1 mM DTT, 0.015% (v/v) Brij 35, 10% (v/v) glycerol; pH 6.8) and 10 μl of diluted enzyme dispensed per well (MultiDrop^{384}, Labsystems, Finland) resulting in a final assay enzyme concentration of 50 ng/well. The stock substrate was also diluted in assay buffer supplemented with 50 mM ATP, and 10 μl of diluted substrate was dispensed per well (MultiDrop^{384}) resulting in a final assay concentration of 1 μM substrate and 50 μM ATP in a total reaction volume of 30 μl per well. Columns 23 and 24 were utilised for internal plate controls consisting of full reaction wells (DMSO vehicle only), IC_{100} wells (25 μM damnacanthal) and IC_{50} wells (0.2 μM damnacanthal).

The reaction was incubated for 60 min at RT after the plates were briefly centrifuged at 500*g* to settle the reaction volume in the wells. Next 60 μl of STOP buffer (50 mM Tris/HCl, 10 mM EDTA, 0.9% (w/v) NaCl, 0.1% (w/v) BSA, 0.67 nM PT66(Eu)Ab and 2 μg/ml S-APC) was added using the MultiDrop^{384}, plates briefly centrifuged at 500*g* and the emission read on the Victor^2 V (Perkin Elmer Life Sciences-Wallac) at both 665 and 615 nm. Data were expressed as the specific signal of the assay, ΔR and is calculated as:

$$\Delta R = [\text{ratio of sample at } 665/615\text{ nm} - \text{ratio of blank } (IC_{100}) \text{ at } 665/615\text{ nm}] / [\text{ratio of full reaction at } 665/615\text{ nm} - \text{ratio of blank } (IC_{100}) \text{ at } 665/615\text{ nm}] * 10{,}000.$$

3.5. Extraction and isolation

The ground flowers (20.01 g) were packed into a metal column (between two layers of sand) inline with two Waters AP-2 MPLC columns packed with C18 and the solvent pumped through at 2 ml/min. The following gradient was used: H_2O for 30 min, followed by H_2O going to MeOH in 120 min, and lastly MeOH for 30 min.

Sixty fractions were collected, and based on screening results, fractions 51–55 were combined, concentrated and applied to a C18 HPLC column. The following conditions were used: H_2O/0.1% TFA:MeOH/0.1% TFA (3:2) going to H_2O/0.1% TFA:MeOH/0.1% TFA (7:13) in 15 min. Compounds **1** (6.59 mg), **2a:2 b** (12.82 mg) **3**, (28.35 mg) and **4** (7.05 mg) were isolated with retention times of 4.8, 12.2, 6.5 and 5.0 min, respectively.

*3.5.1. 3-(4-Hydroxyphenyl) propionyl choline (**1**)*

Amorphous solid (6.59 mg); UV (EtOH) λ_{max} nm (log ε) 218 (*sh*) (3.52), 279 (2.90); ^1H and ^{13}C NMR data, see Table 1; positive-HRESIMS *m/z* 252.1597 (Calcd. for $[C_{14}H_{22}NO_3]^+$ 252.1594).

*3.5.2. 3,4–Di-O-3-(4-hydroxyphenyl) propionyl-1,5-dihydroxycyclohexanecarboxylic acid: 3,5–di-O-3-(4-hydroxyphenyl) propionyl-1, 4-dihydroxycyclohexanecarboxylic acid (**2a:2b**)*

Gum ~2:1 mixture (12.82 mg); ^1H and ^{13}C NMR data, see Table 2; negative-ESIMS *m/z* 487 $[M - H]^-$, $C_{25}H_{27}O_{10}$.

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