Acronyols A and B, new anti-inflammatory prenylated phloroglucinols from the fruits of *Acronychia crassipetala*

Ritesh Raju, Shintu Mathew, Ahilya Singh, Paul Reddell and Gerald Münch

Department of Pharmacology, Western Sydney University, Sydney, NSW, Australia; QBiotics Group Limited, Yungaburra, QLD, Australia

**ABSTRACT**

Two new phloroglucinols, acronyols A (1) and B (2) along with the four known (3–6) phloroglucinols were identified following anti-inflammatory activity guided fractionation from the fruits of *Acronychia crassipetala* (family Rutaceae). The phloroglucinols (1–6) were evaluated for their inhibitory effects on NO production and downregulation of TNF-α in RAW 264.7 macrophage cell lines.

**1. Introduction**

The genus *Acronychia* (family Rutaceae) comprises approximately 50 species with a geographical distribution extending from South East Asia to Australia and islands of the western Pacific Ocean (Epifano et al. 2013). Members of the genus have been used as traditional medicines throughout this region and a wide range of biologically-active phytochemicals has been isolated from *Acronychia* spp. including acridone alkaloids, flavonoids, acetophenones, coumarins, cinnamic acids, lignans, steroids and triterpenes (Epifano et al. 2013).

Phloroglucinols have been identified as common metabolites in different plant parts of the genus *Acronychia*. For example, structurally similar prenylated phloroglucinols, acronyculatins A–E were isolated from the stem and root bark of *Acronychia pedunculata* (Su et al. 2003). While recently a series of acetophenone monomers with one of the oxidized prenyl rings cyclizing to form either a furan or pyran ring were isolated from the bark of *Acronychia trifoliolata* (Miyake et al. 2016). Mildly cytotoxic...
prenylated acetophenone derivatives were isolated from the leaves of *A. oligophlebia* (Yang et al. 2015; Niu et al. 2018). A more recent investigation into the leaves of *A. pedunculata* afforded anti-proliferative isoprenylated acetophenone-quinolone hybrids (Panyasawat et al. 2021).

Herein we report the isolation, structural elucidation and the anti-inflammatory activity of two new (1–2) and four known (3–6) pholoroglucinols from *Acronychia cras-sipetala*, an understory tree endemic to upland and lower montane rainforests in northeastern Queensland, Australia.

2. Results and discussion

HR(+)ESIMS analysis of Acronyol A (1), revealed a pseudomolecular ion ([M + Na]^+) indicative of a molecular formula C_{16}H_{24}O_{5}Na requiring five double bond equivalents. The 1D and 2D NMR data showed resonances assignable to a methyl ketone (δ_H 2.60, s, 3H), a prenyl group supported by COSY and HMBC correlations (Table S1). The prenyl group [C-1''–C-5''] (a set of two methylenes at δ_H 2.57 and 1.60; gem-dimethyls at δ_H 1.72 and 1.63, 3H each, s, respectively). HMBC correlations observed from the gem-dimethyls and the methoxy 3''-OMe (δ_H 3.28) to the oxyquaternary carbon C-3'' (δ_C 75.1) confirmed the methoxy attached to C-3'' of the prenyl residue. The last resonance was assigned to an upfield aromatic proton at δ_H 6.20, 1H, s. The key diagnostic HMBC correlations from [H-3''] to C-1' (δ_C 105.2), C-2' (δ_C 162.1), C-4' (δ_C 163.7) and C-5' (δ_C 109.4)]; [H_2-1'' (δ_H 2.57) to C-4', C-5' and C-6' (δ_C 163.2) and [H_3-2 to C-1 (δ_C 203.1) and C-1' confirmed the position of attachment of the prenyl residue on the heavily oxygenated aromatic ring system, reminiscent of a phloroglucinol backbone (Table S1). Additional HMBC correlations from the methoxy resonances 2''-OMe (δ_H 3.96) and 4''-OMe (δ_H 3.94) to C-2' (δ_C 162.1) and C-4' (δ_C 163.7) respectively confirmed the presence and location of the methoxy residues on the phloroglucinol backbone. The structure of 1 was assigned as 1-[2'', 4''-bis-methoxy-6''-hydroxy-5''-(3''-methoxy-3''-methylbutyl] acetophenone.

HR(+)ESIMS analysis of Acronyol B (2), revealed a pseudomolecular ion ([M + Na]^+) indicative of a molecular formula C_{15}H_{16}O_{5}Na requiring six double bond equivalents. Early on the structural elucidation stage, it was evident that 2 was structurally similar to 1, at least to the point of functional substitution on the benzene ring. The key differences observed were the presence of an oxymethine H-2'' (δ_H 4.27, δ_C 76.9), and a sp^2 carbon C-4'' (δ_H 4.83, 4.72, δ_C 110.5). The sp^2 protons showed HMBC correlations to C-3'', C-2'' and C-5'' confirming the presence of a 3-methylbut-3-en-2-ol residue (Table S2). The low yield of 2 prevented us from performing a Mosher ester analysis to deduce the stereochemistry of the single chiral centre at C-2'', as a result the absolute stereochemistry was left unassigned. The structure of 2 was assigned as 1-[2'', 4'', 6''-trihydroxy-5''-2''-hydroxy-3''-methyl-but-3''-ene] acetophenone.

Compound 3 was identified as 6-demethylacronylin a minor constituent previously isolated from the bark of *Acronychia laurifolia* (Banerji et al. 1973). Compound 4, identified as 2',6'-dihydroxy-5'-prenyl-4'-prenyloxyacetophenone, was only reported as perhaps one of the constituents having anti-inflammatory and anti-allergy extracts from nettle reported in a patent (Alberte et al. 2010) and as a synthetic analogue (Masao
There were no records of this compound as a natural product isolated from a plant source to the best of our knowledge. Compound 5, a methylated derivative of acronylin was only ever reported as a synthetic intermediate (Jain and Zutshi 1972) and as an alkaline degradation product of a derivative of villosinol (Jayaraman et al. 1980). The presence of compounds 4 and 5 as new natural products from Acronychia crassipetala deserves a noteworthy attention, highlighting the chemical diversity that may be present in other Acronychia sp. Finally, the known compound 6 was identified as the recently reported acetophenone, crassipetalonol A from A. crassipetala (Tran et al. 2020).

The anti-inflammatory activity (Table S3) of the isolated compounds (1–6) was evaluated by observing the LPS and IFN-γ induced NO (nitric oxide) inhibition and TNF-α downregulation in RAW 264.7 macrophage cells.

2',6'-dihydroxy-5'-prenyl-4'-prenyloxyacetophenone (4) exhibited the highest level of potency in terms of NO inhibition, while at the same time being moderately cytotoxic. Interestingly, crassipetalonol A (6) had similar level of potency as (4) but exhibited a much lower level of cytotoxicity, structurally they were very similar with the only difference being the change from the methyl ketone (4) to a methylene hydroxy ketone (6) (Figure 1, Table S3).

In comparison, the new pholoroglucinols, acronyol A (1) which lacked the second isoprene unit and an increased saturation of oxy-methyls on the phloroglucinol and isoprene backbone, displayed moderate anti-inflammatory activity with low levels of cytotoxicity. Acronyol B (2) on the other hand with an altered oxygenated isoprene unit displayed weak anti-inflammatory activity (Figure 1, Table S3). The discovery of these small subset pholoroglucinols from the fruits of A. crassipetala lead to an observed structure activity relationship which could be utilized for a broader series of similar prenylated pholoroglucinols.
3. Experimental

3.1. General experimental procedures

UV spectrum was recorded on a Shimadzu spectrophotometer model UV-2550. Chiroptical measurements ([α]D) were obtained on a Polax-D, ATAGO system polarimeter in a 100 × 2 mm cell at 25 °C. NMR spectra were recorded on a Bruker Ascend 400 and 600 MHz spectrometer (Bruker Biospin GmbH, Germany), in the solvents indicated and referenced to residual 1H signals in deuterated solvents. HRMS was carried out using a Waters Xevo Q-TOF mass spectrometer operating in the positive ESI mode. HPLC was carried out using the Agilent 1290 series.

3.2. Materials

Bovine serum albumin, lipopolysaccharide (LPS) (Salmonella serotype), N-(1-1-napthyl) ethylenediamine dihydrochloride, resazurin sodium 10%, streptomycin, sulfanilamide, tetra methyl benzidine (TMB) and Trypan blue were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Dulbecco’s modified Eagle’s medium (DMEM), Phosphate buffer saline (PBS) foetal bovine serum (FBS) and glutamine were GIBCO brands purchased from Life Technologies (Mulgrave, VIC, Australia). Recombinant IFN-γ and TNF-α ELISA kits were purchased from PeproTech Asia (Rehovot, Israel).

3.3. Plant material

Mature fruits of A. crassipetala T.G. Hartley (Rutaceae) were harvested in January 2016 from a remnant fragment of upland tropical rainforest (17°26′41″S, 145°29′40″E, 990 m) on the Atherton Tablelands, northeastern Queensland, Australia. A voucher specimen of the original collection is held in the QBiotics herbarium (Specimen # YA1505).

3.4. Extraction and isolation

The fruits of A. crassipetala (350 g) was crushed using a hand blender and extracted with absolute EtOH. The crude extract was then dried in vacuo giving a viscous dark brown syrup (36.7 g). A portion of this extract (25.8 g) was subsequentially partitioned between water (5.8 g) and DCM (16.8 g). The DCM fraction was suspended in MeOH and was then later subjected to semi-preparative HPLC using a C8 (Agilent, 5 μm, 250 × 9.4 mm) column eluting at 2 mL/min from 10% MeCN/H2O to 100% MeCN (with a constant 0.01% TFA modifier) over 20 mins and held for a further 15 mins, to yield 1 (tR = 22.1 min, 1.1 mg), 2 (tR = 19.2 min, 1.3 mg), 3 (tR = 20.8 min, 3.5 mg), 4 (tR = 27.2 min, 3.8 mg), 5 (tR = 23.6 min, 1.0 mg) and 6 (tR = 25.3 min, 2.6 mg) successively.

3.4.1. Acronyol A (1)

White powder; UV (MeOH) λmax (log ε): 200 (4.42), 213 (4.36), 290 (4.22), 330 (3.21) nm; 1H and 13C NMR data, see Table S1 and Figures S1–S4; HRMS [M + Na]+ m/z 319.1528 (calcd for C16H24O5Na, 319.1521).
3.4.2. Acronyol B (2)
Yellow oil; $[x]_{D}^{25} +42$ (c 0.01, MeOH); UV (MeOH) $\lambda_{\text{max}}$ (log $\epsilon$): 205 (4.46), 220 (4.38), 290 (4.20), 330 (3.20) nm; $^1$H and $^{13}$C NMR data, see Table S2 and Figures S5–S8; HRMS [M + Na]$^+$ m/z 275.0883 (calcd for C$_{13}$H$_{16}$O$_{5}$Na, 275.0895).

3.5. Maintenance of N11 microglia and RAW 264.7 macrophages
Cells were grown in 175 cm$^2$ flasks on DMEM containing 5% foetal bovine serum (FBS) that was supplemented with penicillin (100 $\mu$g/ml), streptomycin (100 $\mu$g/ml) and L-glutamine (2 mM). The cell line was maintained in 5% CO$_2$ at 37°C, with media being replaced every 3–4 days. Once cells had grown to confluence in the culture flask, they were removed using a rubber policeman, as opposed to using trypsin, which can remove membrane-bound receptors.

3.6. Pro-inflammatory activation of cells
For assays, 90 $\mu$L of each concentration of samples (8 concentrations made by serial dilution in DMEM) were added an hour prior to addition of 10 $\mu$L of activator. A combination of 10 $\mu$g ml$^{-1}$ LPS and 10 U ml$^{-1}$ (1 unit = 0.1 ng/mL) IFN-$\gamma$, were used for activation. After activation, the cells were incubated for 24 h at 37°C and then NO, TNF-$\alpha$ and cell viability was determined. Unactivated cells (exposed to media alone) were used as negative control and activated cells as positive control.

3.7. Determination of nitrite (as a measure of nitric oxide production) by the Griess assay
Nitric oxide was determined by Griess reagent quantification of nitrite. Griess reagent was freshly made up of equal volumes of 1% sulfanilamide and 0.1% napthyethylene-diamine in 5% HCl. From each well, 50 $\mu$L of supernatant was transferred to a fresh 96-well plate and mixed with 50 $\mu$L of Griess reagent and measured at 540 nm in a POLARstar Omega microplate reader (BMG Labtech, Mornington, Australia). The concentration of nitrite was calculated using a standard curve with sodium nitrate (0–500 $\mu$M), and linear regression analysis.

3.8. Determination of TNF-$\alpha$ by ELISA
The diluted supernatants were used for determination of TNF-$\alpha$ using a commercial sandwich ELISA (Peprotech) according to the manufacturer’s protocol with small modifications. In brief, the capture antibody was used at a concentration of 1.5 $\mu$g ml$^{-1}$ in PBS (1.9 mM NaH$_2$PO$_4$, 8.1 mM Na$_2$HPO$_4$, 154 mM NaCl) (pH 7.4). Serial dilutions of TNF-$\alpha$ standard from 0 to 10,000 pg mL$^{-1}$ in diluent (0.05% Tween-20, 0.1% BSA in PBS) were used as internal standard. TNF-$\alpha$ was detected with a biotinylated second antibody and an Avidin peroxidase conjugate with TMB as detection reagent. The colour development was monitored at 655 nm, taking readings every 5 min. After about 30 min the reaction was stopped using 0.5 M sulphuric acid and the absorbance was measured at 450 nm using a POLARstar Omega microplate reader (BMG Labtech,
Mornington, Australia) and expressed as a percentage of that in control cells after conversion of the concentrations by using a standard curve constructed with defined concentrations of TNF-α. Curve fitting of this standard curve and extrapolation of experimental data were performed using non-linear regression analysis.

3.9. Determination of cell viability by the alamar blue assay

100 μl of Alamar Blue solution (10% Alamar Blue (Resazurin) in DMEM media) was added to each well, incubated at 37 °C for 2 h. After incubation, fluorescence intensity was measured with the microplate reader (excitation at 530 nm and emission at 590 nm) and results were expressed as a percentage of the intensity of that in control cells, after background fluorescence was subtracted.

3.10. Statistical analysis

Data calculations were performed using MS-Excel 2010 software. IC₅₀ values were obtained by using the sigmoidal dose–response function in GraphPad Prism. The results were expressed as mean ± standard deviation (SD).

4. Conclusion

We isolated six phloroglucinols (1–6) from the fruits of A. crassipetala. Acronyol A (1) and B (2) are previously undescribed while this is the first report of the occurrence of phloroglucinols 4 and 5 in this species. All compounds displayed weak to moderate anti-inflammatory activity (IC₅₀ values as low as 14.7 and 37.8 μM) in terms of downregulation of NO and TNF-α production respectively.

Acknowledgements

The authors would like to acknowledge Dr. Scott Willis (Biomedical Magnetic Resonance Facility Manager, Western Sydney University) for providing his technical expertise in the NMR experiments performed. We also would like to acknowledge the Mass Spectrometry Facility (MFS) of Western Sydney University for access to its instrumentation and grateful for the assistance of Mr Meena Mikhael.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The author(s) reported there is no funding associated with the work featured in this article.

References

Alberte RS, Roschek WP, Li D. 2010. Antiinflammatory and anti-allergy extracts from nettle. US Patent. US 2010/0009927 A1.
Banerji J, Rej RN, Chatterjee A. 1973. 6-demethylacronylin, a minor phenolic constituent of Acronychia laurifolia BL. (Rutaceae). Indian J Chem. 11:693–694.

Epifano D, Fiorito S, Genovese S. 2013. Phytochemistry and pharmacognosy of the genus Acronychia. Phytochemistry. 95:12–18.

Jain AC, Zutshi MK. 1972. Nuclear prenylation of 2-O-methylphloracetophenone. Synthesis of preremirol, acronylin, evodinol and isoevodinol. Tetrahedron. 28(22):5589–5593.

Jayaraman I, Ghanim A, Khan HA. 1980. A new prenylated flavanone from Tephrosia villosa. Phytochemistry. 19(6):1267–1268.

Masao T, Makoto K, Yasuhiko K. 1994. Regioselective synthesis of prenylphenols. Synthesis of naturally occurring 4′-alkenyloxy-2′,6′-dihydroxy-3′-(3-methyl-2-butenyl) acetophenones. Chem Lett. 7:1203–1206.

Miyake K, Suzuki A, Goto M, Newman DJ, O’Keefe BR, Morris-Natschke SL, Lee K-H, Nakagawa-Goto K. 2016. Acetophenone monomers from Acronychia trifoliata. J Nat Prod. 79(11):2883–2889.

Niu Q-W, Chen N-H, Wu Z-N, Luo D, Li YY, Zhang Y-B, Li Q-G, Li Y-L, Wang G-C. 2018. Isolation and identification of new prenylated acetophenone derivatives from Acronychia oligophlebia. Nat Prod Res. 33(15):2230–2235.

Panyasawat P, Wisetsai A, Lekphrom R, Senawong T, Schevenels FT. 2021. Acroquinolones A and B, two polyphenolic isoprenylated acetophenone-quinolone hybrids with anti-proliferative activities from Acronychia pedunculata (L.) Miq. Nat. Prod. Res. 1–10. doi:10.1080/14786419.2021.1922405.

Su R-C, Kuo P-C, Wang M-L, Liou M-J, Damu AG, Wu T-S. 2003. Acetophenone derivatives from Acronychia pedunculata. J Nat Prod. 66(7):990–993.

Tran TD, Olsson MA, McMillan DJ, Cullen JK, Parson PG, Reddell PW, Ogbourne SM. 2020. Potent antibacterial prenylated acetophenones from the Australian endemic plant Acronychia crassipetala. Antibiotics. 9(8):487–495.

Yang X, Zhang Y-B, Wu Z-N, Zhang X-Q, Jiang JW, Li Y-L, Wang G-C. 2015. Six new prenylated acetophenone derivatives from the leaves of Acronychia oligophlebia. Fitoterapia. 105:156–159.