Pim2 Inhibitors from the Papua New Guinean Plant *Cupaniopsis macropetala*[⊥]

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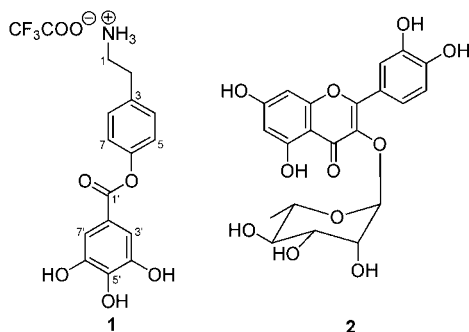
Bioassay-guided fractionation of an organic extract from the leaves of *Cupaniopsis macropetala* resulted in the isolation of a new alkaloid, galloyl tyramine (**1**), together with the known flavonoid glycoside quercitrin (**2**). The structure of **1** was determined following 1D and 2D NMR, IR, UV, and MS data analysis. Compounds **1** and **2** displayed IC_{50} values of 161 and 25 μM, respectively, in a Pim2 enzyme assay.

The Pim family of cytoplasmic serine/threonine kinases consists of three highly homologous genes, *Pim1*, *Pim2*, and *Pim3*.[1,2] These enzymes are increasingly being recognized as important regulators of apoptosis and cellular metabolism. Pim overexpression has been reported in various cancers, including B cell lymphoma, chronic lymphocytic leukemia, prostate cancer, and acute myelogenous leukemia.[3] Pim kinases have also been shown to be activated in retroviral-induced lymphomas, e.g., Moloney murine leukemia virus.[4,5] Thus, pharmacological inhibition of these Pim kinases could have applications in the treatment of diseases such as cancer, inflammatory disorders, and ischemic diseases.[3]

High-throughput screening of ~35 000 biota extracts led to the discovery that an extract from the leaves of the rainforest tree *Cupaniopsis macropetala* Radlk. (Sapindaceae) showed potent activity in a Pim2 enzyme assay. Bioassay-guided fractionation of an organic extract resulted in the isolation of a new alkaloid, galloyl tyramine (**1**), along with the previously reported flavonoid glycoside quercitrin (**2**). Herein we report the isolation and structure elucidation of galloyl tyramine (**1**) along with the Pim2 inhibitory activity of both natural products.



The air-dried and ground leaves of *C. macropetala* were sequentially extracted with CH_2Cl_2 and MeOH. Both extracts were combined and chromatographed using reversed-phase C_{18}-bonded silica HPLC to yield galloyl tyramine (**1**, 82.5 mg, 1.914% dry wt) and quercitrin (**2**, 43 mg, 0.998% dry wt).

The TFA salt of galloyl tyramine (**1**) was isolated as a brown gum and was assigned the molecular formula $C_{17}H_{16}F_3NO_7$ on the basis of (+)-HRESIMS and NMR data. The ^{1}H NMR spectrum of **1** displayed six downfield exchangeable resonances [δ_H 9.38 (s, 2H), 9.12 (s, 1H), and 7.84 (brs, 3H)], two aromatic resonances [δ_H 7.32 (d, $J = 8.4$ Hz, 2H), 7.17 (d, $J = 8.4$ Hz, 2H)] indicative of a 1,4-disubstituted benzene ring, one aromatic singlet [δ_H 7.08 (s, 2H)], and two mutually coupled methylenes [δ_H 2.88 (brt, $J =$ 7.8 Hz, 2H) and 3.07 (brs, 2H)]. The ^{13}C NMR spectrum of **1** contained only 11 resonances, nine of which appeared between δ_C 109 and 165. Analysis of the gHSQC data enabled all protonated carbons to be assigned. A HMBC correlation from δ_H 7.32 (H-4, H-8) to the ethylene carbon at δ_C 32.4 (C-2) and a strong ROESY correlation between H-4/H-8 and H-2 indicated that the ethylene moiety was attached to C-3 of the 1,4-disubstituted benzene ring. HMBC correlations from δ_H 2.88 (H-2) to the quaternary carbon at δ_C 134.5 (C-3) and the methine carbon at δ_C 129.6 (C-4, C-8) further confirmed this assignment. A strong COSY correlation between δ_H 3.07 (H-1) and the exchangeable signal at δ_H 7.84 suggested a protonated terminal amine group was attached to C-1 (δ_C 39.9). HMBC correlations from both δ_H 7.32 (H-4, H-8) and 7.17 (H-5, H-7) to a quaternary oxygenated carbon at δ_C 149.6 (C-6) indicated that **1** contained a tyramine residue. Thus, the remaining portion of **1** consisted of $C_7H_5O_4$. Due to the symmetry of this unassigned unit, as inferred by the NMR data, this moiety could only be a phloroglucinol carboxylic acid or gallic acid derivative. HMBC correlations from the remaining aromatic methine at δ_H 7.08 (H-3′, H-7′) to two oxygenated carbons at δ_C 145.7 (C-4′, C-6′) and δ_C 139.2 (C-5′) and a strong $^3J_{CH}$ correlation to a carbonyl at δ_C 164.6 (C-1′) confirmed the presence of a gallic acid moiety in **1**. Two- and three-bond HMBC correlations from the phenolic hydroxy resonances at δ_H 9.38 (s, 2H) to carbons at δ_C 109.1 (C-3′, C-7′), 145.7 (C-4′, C-6′), and 139.2 (C-5′) provided further evidence of the gallic acid structure. Although no ROESY or HMBC correlations were observed between the gallic acid and tyramine substructures of **1**, these systems by default were linked via an ester group. A strong absorption in the IR spectrum at ν_{max} 1710 cm^{-1} confirmed this linkage and allowed the structure of **1** to be assigned to galloyl tyramine.

The known flavonoid glycoside quercitrin (**2**) was identified by comparison of NMR and optical rotation data with literature values.[6]

Galloyl tyramine and quercitrin were tested for inhibition activity against Pim2. Both compounds **1** and **2** proved to be weak inhibitors of the kinase with IC_{50} values of 161 and 25 μM, respectively. Interestingly, several non-glycosylated flavonoids related to **2** such as quercetagetin, gossypetin, and myricetin have been shown recently to inhibit Pim1 activity.[1] Quercetagetin proved to be a highly selective inhibitor of Pim1 compared to Pim2 and seven other serine-threonine kinases.[1]

Plant metabolites containing galloyl derivatives are relatively common and have been shown to display a number of different biological activities, although no Pim activity has been reported.[7]

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Galloyl-, *m*-digalloyl-, and *m*-trigalloyl L-tyrosine from the young leaves of the rainforest plant *Inga laurina* have been proposed recently to provide this plant with a chemical defense against herbivores during the vulnerable expansion stage of leaf development.[8] Green tea catechins, such as epigallocatechin gallate, have been shown to exhibit antimicrobial activities, which is believed to arise from their inhibition of bacterial DNA gyrase.[9]

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a JASCO P-1020 polarimeter. UV and IR spectra were recorded on a Camspec M501 spectrophotometer and a Bruker Tensor 27 spectrometer, respectively. NMR spectra were recorded at 30 °C on either a Varian Unity INOVA 500 MHz or Varian NMR System 600 MHz spectrometer. The latter spectrometer was equipped with a triple resonance cold probe. The ^{1}H and ^{13}C chemical shifts were referenced to the proto-deutero solvent peak for DMSO-d_6 at δ_H 2.49 and δ_C 39.5. LRESIMS were recorded on a Waters ZQ mass spectrometer. HRESIMS were recorded on a Bruker Daltonics Apex III 4.7e Fourier-transform mass spectrometer, fitted with an Apollo API source, funded by ARC LIEF (2002). A Waters 600 pump equipped with a Waters 996 PDA detector and a Waters 717 autosampler were used for HPLC. A ThermoElectron C_{18} Betasil 5 μm 143 Å column (21.2 mm × 150 mm) was used for semipreparative HPLC separations. All solvents used for chromatography, UV, optical rotations, and MS were Laboratory-Scan HPLC grade, and the H_2O was Millipore Milli-Q PF filtered.

Plant Material. The leaves of *Cupaniopsis macropetala* (Sapindaceae) were collected during January of 2002 at Edevu, Central Province, Papua New Guinea. A voucher sample (3002102P01779) has been lodged at Biodiversity Research Ltd., Port Moresby, Papua New Guinea. The plant material was collected according to the UN Convention on Biological Diversity (1993) with a benefit sharing agreement in place.

Extraction and Isolation. The air-dried and ground leaves of *C. macropetala* (4.31 g) were extracted with CH_2Cl_2 (1 L) and MeOH (1 L). The solvents were combined then evaporated to yield a dark brown residue (1.0 g), which was preadsorbed to C_{18}-bonded silica (5 g). The preadsorbed material was packed into a stainless steel cartridge (10 × 20 mm) and the cartridge attached to a C_{18} semipreparative HPLC column. A linear gradient from H_2O containing 1% TFA to MeOH containing 1% TFA at a flow rate of 9 mL/min over 35 min was run, then isocratic conditions of MeOH containing 1% TFA were maintained for a further 25 min at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected from time = 0 min. Fraction 33 contained galloyl tyramine as its TFA salt (**1**, 82.5 mg, 1.914% dry wt), and fraction 44 was quercitrin (**2**, 43 mg, 0.998% dry wt).

TFA Salt of Galloyl Tyramine (1): brown oil; UV (MeOH) λ_{max} (log ϵ) 212 (4.68), 272 (4.30) nm; IR ν_{max} (film) 3500–3000, 1710, 1679, 1610, 1534, 1509, 1447, 1352, 1195, 1137, 1042, 1024, 993, 958, 870, 827, 801, 761, 722 cm^{-1}; ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.88 (2H, brt, J = 7.8 Hz, H-2), 3.07 (2H, brs, H-1), 7.08 (2H, s, H-3′, H-7′), 7.17 (2H, d, J = 8.4 Hz, H-5, H-7), 7.32 (2H, d, J = 8.4 Hz, H-4, H-8), 7.84 (3H, brs, 1-NH_3^+), 9.12 (1H, s, OH-5′), 9.38 (2H, s, OH-4′, OH-6′); ^{13}C NMR (125 MHz, DMSO-d_6) δ 32.4 (C-2), 39.9 (C-1), 109.1 (C-3′, C-7′), 118.2 (C-2′), 122.0 (C-5, C-7), 129.6 (C-4, C-8), 134.5 (C-3), 139.2 (C-5′), 145.7 (C-4′, C-6′), 149.6 (C-6), 164.6 (C-1′); (+)-LRESIMS *m/z* 290 [M-CF_3COO]$^+$; (+)-HRESIMS *m/z* 290.1014 ($C_{15}H_{16}NO_5$ [M − CF_3COO]$^+$ requires 290.1023).

Quercitrin (2): yellow gum; $[\alpha]^{19}_D$ −135 (*c* 0.246, MeOH), lit. $[\alpha]^{21}_D$ −157 (*c* 0.670, MeOH).[6]

Inhibition of Pim2 Kinase Phosphorylation of Biotinylated-BAD Substrate. Commercially available AlphaScreen[10] assay kits (PerkinElmer) were utilized to measure the Pim2-mediated phosphorylation of a biotinylated BAD (Bt-BAD) peptide (RSRHSSYPAGT). Enzyme, substrate, and antibody were kindly supplied by AstraZeneca.

Two microliters of natural product extract and controls (10% DMSO or 0.5 M EDTA) was added to a 384-well microtiter plate. Enzyme and substrate solutions were prepared in assay buffer (50 mM HEPES, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 420 μg/mL BSA, 0.05% Tween-20, and 10 mM NaCl) and incubated separately for 1 h at rt. Five microliters of 62.1 pM Pim2 enzyme and 5 μL of assay buffer containing 0.39 μg/mL Bt-BAD and 48 μM adenosine triphosphate (ATP) were added. The reaction was then incubated for 70 min at rt and stopped by the addition of 5 μL of stop solution containing 0.51 nM Antiphospho-BAD antibody, 0.04 mg/mL antimouse IgG acceptor beads, and 0.04 mg/mL streptavidin donor beads in stop buffer (30 mM EDTA, pH 8.0, 15 mM HEPES, pH 7.5, and 0.5 mg/mL BSA). Following addition of the stop solution, the assay plates were sealed and incubated at rt in the dark overnight. Plates were read using a Wallac Envision or AlphaFusion, with a 680 nm excitation filter and 520–650 nm emission filter.

Compounds were assayed for inhibition over 11 concentrations from 666 μM to 3.3 nM in duplicate on two separate days. Staurosporine was used as the positive control for this assay and gave an average IC_{50} of 28 nM. IC_{50} values were determined using GraphPad Prism Version 4.03.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra and (+)-LRESIMS for the TFA salt of galloyl tyramine (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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