Diarylheptanoid from *Pleuranthodium racemigerum* with *in Vitro* Prostaglandin E_2 Inhibitory and Cytotoxic Activity

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Bioactivity-guided fractionation of an ethanolic extract of the rhizome of *Pleuranthodium racemigerum*, a tropical Zingiberaceae species from Northeastern Australia, resulted in the isolation and structural elucidation of 1-(4″-methoxyphenyl)-7-(4′-hydroxyphenyl)-(*E*)-hept-2-ene (**1**), a new diarylheptanoid related to curcumin. Compound **1** was a fairly potent inhibitor of prostaglandin E_2 production in 3T3 murine fibroblasts ($IC_{50} \approx 34$ μM) and also displayed moderate cytotoxicity against this cell line ($IC_{50} = 52.8$ μM). The compound also demonstrated cytotoxic activity against the P388D1 murine lymphoblast cell line ($IC_{50} = 117.0$ μM) and four human cell lines: Caco-2 colonic adenocarcinoma ($IC_{50} = 44.8$ μM), PC3 prostate adenocarcinoma ($IC_{50} = 23.6$ μM), HepG2 hepatocyte carcinoma ($IC_{50} = 40.6$ μM), and MCF7 mammary adenocarcinoma ($IC_{50} = 56.9$ μM). The cytotoxicity of compound **1** closely resembled that of curcumin, in terms of both IC_{50} values and dose−response curves.

The ginger family, Zingiberaceae, is a monocotyledenous family in the order Zingiberales. The family comprises 48 genera with a total of over 1200 species. The family is essentially tropical in distribution and is richly represented in the Indomalesian flora, i.e., from India to New Guinea.[1] In Australia, the Zingiberaceae is represented by 14 native species from eight genera. In addition, seven introduced species from five genera also occur.[2] Many plants belonging to the family have a history of medicinal use in systems of traditional medicine. Best known are ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*), both of which have been the subject of substantial pharmacological and clinical investigations over the last three decades, but many lesser known species are also used, mostly in tropical Asia, where the majority are native. Several species in the family are also important spices.

Zingiberaceae species often produce essential oils in their thickened rhizomes, as well as nonvolatile compounds. Representing the latter, diarylheptanoids are common in the family, the best known example being curcumin (diferuloylmethane), which has been studied extensively for its wide-ranging pharmacological properties, which include antioxidant, anti-inflammatory, antitumor, and chemopreventive activities.[3−5] Related diarylheptanoids are known from the genera *Curcuma*,[6] *Zingiber*,[7−10] *Alpinia*,[11] and *Renealmia*.[12]

Diarylheptanoids possess potent anti-inflammatory properties. Numerous studies have indicated that the key to the anti-inflammatory activity of curcumin is its ability to inhibit the activation of the transcription factor NFκB, which plays a critical role in the transcriptional regulation of pro-inflammatory gene expression, but multiple other targets have been identified.[13−15] *In vitro* data support potential anti-inflammatory activity for at least some other diarylheptanoids, e.g., yakuchinones A and B isolated from *Alpinia* species.[16−18]

The genus *Pleuranthodium* comprises some 23 species distributed in New Guinea and tropical Australia.[1] In Australia, *P. racemigerum* (F. Muell.) R. M. Smith (syn. *Alpinia racemigera* F. Muell., *Psychanthus racemiger* (F. Muell.) R. M. Smith) occurs in the Wet Tropics region of Far North Queensland and was the focus of the present work. No information about the chemistry or pharmacology of this species has been published previously.

Table 1. Inhibition of PGE_2 Production in 3T3 Murine Fibroblast Cells by Fractions of *Pleuranthodium racemigera* Extract

	percent inhibition (mean ± SD, $n = 3$)		
fraction no.[a]	1 μg/mL[b]	5 μg/mL[b]	10 μg/mL[b]
1	−35 ± 3	−20 ± 3	−33 ± 3
2	4 ± 0	17 ± 2	27 ± 1
3	10 ± 1	24 ± 6	28 ± 3
4	15 ± 1	41 ± 9	47 ± 4
5	−1 ± 0	19 ± 2	−4 ± 0
6	−5 ± 0	13 ± 1	−11 ± 2
aspirin 9 μg/mL (0.05 mM)[a,b]		30 ± 8	

[a] Fractions and reference compounds were dissolved in DMSO at a concentration of 10 mg/mL prior to being assayed. [b] Concentrations are final concentrations of test substance in assay well.

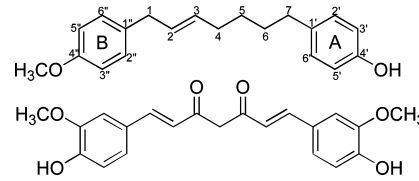

Figure 1. Structure of compound **1** (top) and curcumin (bottom).

An ethanolic extract of the dried rhizome of *P. racemigerum* was subjected to bioactivity-guided fractionation by preparative HPLC using inhibition of prostaglandin E_2 (PGE_2) production in 3T3 murine fibroblasts to guide the fractionation. The 29 fractions obtained initially were recombined into six fractions; of these, fraction 4 caused the most potent inhibition of PGE_2 production (Table 1). Analysis by LC-MS proved this fraction to consist of a pure compound, compound **1** (Figure 1), which was isolated as a honey-colored, viscous oil. HREIMS and ESIMS experiments yielded a molecular formula of $C_{20}H_{24}O_2$. The ^{1}H NMR spectrum showed signals in the region 6.7−7.2 ppm, representing the aromatic protons of two *para*-disubstituted rings of an AA′BB′ spin system

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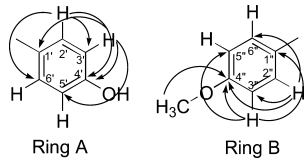

Figure 2. Heteronuclear multiple bond correlations in the two aromatic rings in compound **1**.

Table 2. IC_{50} (μM) Values for Cytotoxic Activity of Compound **1** and Curcumin against Five Cancer Cell Lines[a]

cell line	compound 1	curcumin
Caco-2	44.8 (32.6−61.8)	48.6 (38.9−60.4)
PC3	23.6 (20.6−26.9)	22.7 (17.7−28.9)
HepG2	40.6 (28.0−58.9)	43.8 (25.6−75.2)
MCF7	56.9 (45.3−71.1)	51.5 (34.8−76.0)
P388D1	117.0 (53.4−255.6)	75.7 (65.9−86.8)

[a] 95% confidence intervals are shown in brackets.

(rings A and B, Figure 2). The HSQC data showed that H-2′/H-6′ and H-2″/H-6″ were directly correlated to the carbon signal at 129.6 ppm, and H-3′/H-5′ and H-3″/H-5″ protons to the carbon signals at 115.3 and 114.0 ppm, respectively. A singlet at 3.82 ppm (3H) was observed in the ^{1}H NMR spectrum, consistent with a methoxy substituent, and the $^3J_{CH}$ correlation of the methoxy protons to the quaternary carbon at 158.0 ppm (C-4″) placed the methoxy group at C-4″. A $^3J_{CH}$ correlation of H-3′/H-5′ protons was observed to another downfield quaternary carbon at 153.8 ppm, placing the carbon at the C-4′ position with a hydroxyl substituent. H-3′/H-5′ and H-3″/H5″ protons showed $^3J_{CH}$ correlations to the quaternary carbon signals at 135.2 ppm (C-1′) and 133.4 ppm (C-1″), respectively, thus completing the carbon assignments for the two aromatic ring systems. The proton signals at 5.55 ppm (H-2) and 5.51 ppm (H-3) indicated olefinic protons, and the *J*-value of 15.2 Hz suggested that the protons were coupled to each other in an *E* configuration. Five methylene proton signals (H-1, H-4, H-5, H-6, and H-7) were also present in the region from 1.4 to 3.3 ppm, and the COSY experiment showed that H-1 and H-4 were vicinal to H-2 and H-3, respectively. H-5 also showed $^3J_{HH}$ correlation to H-4 and H-6, and H-6 to H-7. HMBC data showed $^2J_{CH}$ correlation of H-1 to the quaternary carbon at 133.4 ppm (C-1″), which connected C-1 to C-1″. Likewise, H-7 showed $^2J_{CH}$ correlation to the quaternary carbon at 135.1 ppm (C-1′), linking C-7 to C-1′. The APCI-MS data showed an $(M + 1)^+$ value of 297.2, which is consistent with a molecular formula of $C_{20}H_{24}O_2$. On the basis of these data, the structure of compound **1** was confirmed as 1-(4″-methoxyphenyl)-7-(4′-hydroxyphenyl)-(*E*)-hept-2-ene, a new diarylheptanoid related to curcumin.

Compound **1** was a fairly potent inhibitor of PGE_2 production in 3T3 murine fibroblasts ($IC_{50} \approx 34$ μM). It also displayed moderate cytotoxicity against this cell line ($IC_{50} = 52.8$ μM after incubation for 3 h) and thus appears to lack selectivity. The compound was also tested for cytotoxic activity against one other murine cell line (P388D1 lymphoblast) and four human cell lines (Caco-2 colonic adenocarcinoma, PC3 prostate adenocarcinoma, HepG2 hepatocyte carcinoma, and MCF7 mammary adenocarcinoma). The cytotoxicity of compound **1** closely resembled that of curcumin, in terms of both IC_{50} values (Table 2) and dose−response curves (Figure 3).

This is the first report concerning the chemistry and pharmacology of *P. racemigerum*. The anti-inflammatory and cytotoxic activities reported for the diarylheptanoid (**1**) suggest this new compound warrants further investigation. Further work should focus on any selective activity that the compound may possess.

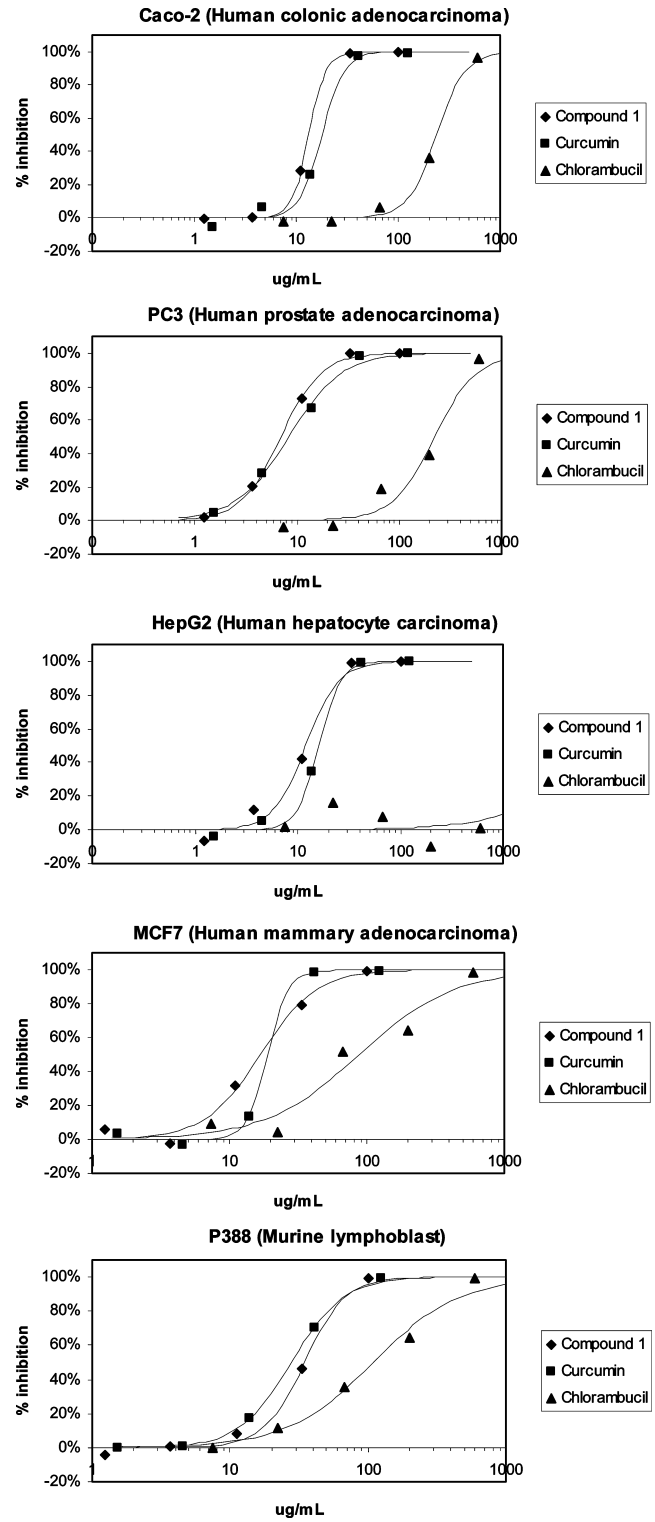

Figure 3. Cytotoxic activity of compound **1** and curcumin against four human and one murine cancer cell line.

Experimental Section

General Experimental Procedures. Fractionation was carried out on a Gilson Preparative HPLC system with binary pump, Rheodyne injector, and dual-wavelength detector and fitted with a Gilson FC204 fraction collector using an Altima C-18 5 μm column (150 × 22 mm i.d.; Alltech, KY). Fractions were analyzed on an Agilent (Palo Alto, CA) 1100 Series LC-MSD system with binary pump, autoinjector, and photodiode array and mass detectors, and fitted with a Phenomenex (Torrance, CA) Luna 5 μm (150 × 4.6 mm i.d.) C-18 column.

UV absorbance data were obtained on a Hewlett-Packard 8453 spectrophotometer (Palo Alto, CA) controlled by UV−visible ChemStation software (Rev. A.0803; Dayton, OH). Compound **1** was dissolved in MeOH, and absorbance data were acquired in the range 190−1100 at 1 nm intervals in five replicates.

NMR spectra were obtained using a Bruker AVANCE DRX500 (^{1}H at 500.13 MHz; ^{13}C at 125.77 MHz; 5 mm QNI probe) spectrometer with XWin-NMR software. The ^{1}H and ^{13}C NMR spectra were recorded using $CDCl_3$ with the residual solvent peaks as reference (7.27 ppm for ^{1}H and 77.2 ppm for ^{13}C). The chemical shifts were expressed in parts per million (ppm) as δ values and the coupling constants (J) in hertz (Hz). All experiments were carried out using the Bruker pulse programs.

HREIMS experiments were conducted on a Kratos MS25 RFA instrument via a direct insertion probe at 70 eV and source temperature of 200 °C. Perfluorokerosene (PFK) was used as reference. ESIMS experiments were conducted on a Finnigan MAT 900 XL-Trap instrument with a Finnigan API III electrospray source, using MeOH as the solvent and polypropylene glycol and polyethylene glycol as references for accurate mass data, acquired by electric sector scan.

Plant Material. *Pleuranthodium racemigerum* was collected in a rainforest (828 m above sea level) in the Wet Tropics region of North Queensland in Gillies Range (S 17°13′; E 145°40′) under a permit issued by the Queensland Environmental Protection Agency (permit no. WISP02945705). It was identified by a field botanist, Mr. Rigel Jensen, Malanda, Queensland, and one of the authors (H.W.). Voucher specimens have been deposited in the Medicinal Plant Herbarium at Southern Cross University (NCM05-047) and the Queensland Herbarium (AQ736263).

Extraction and Isolation. Fresh rhizomes were cleaned, sliced, and dried at 40 °C. The dried rhizomes were ground to a coarse powder in a Waring blender, and 10.0 g was extracted with 4 parts (by mass) EtOH. The plant material was steeped for 24 h in a stoppered conical flask while being agitated on a Bioline BL 4236 orbital shaker (Edwards Instrument Company, Australia). The resulting extract was filtered through a Quickfit glass filter and stored at −20 °C. Prior to fractionation, the extract was dried under vacuum on a Büchi R-114 rotavapor (Switzerland) with the water bath temperature at 45 °C. The resulting extract, which was oily, was redissolved in 91% aqueous MeOH and partitioned with *n*-hexane in a separating funnel. The MeOH phase was dried under vacuum, yielding 700 mg of dry extract. Prior to testing in bioassays a small quantity of extract was dried under a nitrogen stream and redissolved to a known concentration. The MeOH extract residue was redissolved in 3.5 mL of MeCN with a few drops of H_2O and fractionated on a Gilson Preparative HPLC system as described in the previous section. Mobile phase A consisted of HPLC-grade H_2O, and mobile phase B consisted of HPLC-grade MeCN; both contained 0.05% TFA. The flow rate was 20 mL/min, and elution was performed using a linear gradient of 30% to 95% mobile phase B over 20 min followed by a 5 min hold of 95% mobile phase B. UV−vis detection was at 210 and 360 nm. Twenty-nine fractions were collected at 1 min intervals, commencing at 1 min. Four 1 mL injections of the sample were fractionated and the fractions dried on a rotational vacuum concentrator for 48 h at −88 °C and 0.140 mbar (Christ Alpha 2-4, Osterode, Germany). These fractions were recombined to give six fractions that were tested in the PGE_2 assay (Table 1). The mass of fractions 1−6 were 11.9, 14.7, 20.4, 217.4, 26.5, and 10.2 mg, respectively. The dry extract and fractions were stored at −20 °C until use.

1-(4″-Methoxyphenyl)-7-(4′-hydroxyphenyl)-(*E*)-hept-2-ene (1): honey-colored oil; UV (MeOH) λ_{max} (log ε) 203 (4.40), 225 (4.24), 278 (3.54) nm; ^{1}H NMR, δ 7.11 (2H, d, J = 8.7 Hz, H-2″, H-6″), 7.04 (2H, d, J = 8.5 Hz, H-2′, H-6′), 6.86 (2H, d, J = 8.7 Hz, H-3″, H-5″), 6.76 (2H, d, J = 8.5 Hz, H-3′, H-5′), 5.55 (1H, dt, J = 15.2, 6.4 Hz, H-2), 5.51 (1H, dt, J = 15.2, 6.4 Hz, H-3), 3.82 (3H, s, OCH_3), 3.29 (2H, d, J = 6.4 Hz, H-1), 2.55 (2H, t, J = 7.8 Hz, H-7), 2.07 (2H, dt, J = 7.3, 6.4 Hz, H-4), 1.61 (2H, dt, J = 7.8, 7.7 Hz, H-6), 1.43 (2H, tt, J = 7.7, 7.3 Hz, H-5); ^{13}C NMR, δ 158.0 (C, C-4″), 153.8 (C, C-4′), 135.1 (C, C-1′), 133.4 (C, C-1″), 131.6 (CH, C-3), 129.6 (5 × CH, C-2, C-2′, C-6′, C-2″, C-6″), 115.3 (2 × CH, C-3′, C-5′), 114.0 (2 × CH, C-3″, C-5″), 55.0 (CH_3, Ar-OCH_3), 38.3 (CH_2, C-1), 35.0 (CH_2, C-7), 32.5 (CH_2, C-4), 31.4 (CH_2, C-6), 29.2 (CH_2, C-5); APCIMS m/z 295.2 $[M - 1]^+$; ESIMS m/z 319 $[M + Na]^+$; HREIMS m/z 319.1683 $[M + Na]^+$ (calcd for $C_{20}H_{24}O_2Na$, 319.1674).

Prostaglandin E_2 Assay. This assay was carried out in 96-well plates using Swiss albino mouse embryo fibroblast cells 3T3 (American Type Culture Collection (ATTC), Manassas, VA) and a PGE_2 enzyme immuno-assay kit (Prostaglandin E_2 EIA kit−monoclonal, Cayman Chemical Company, Ann Arbor, MI, catalog no. 514010). Murine 3T3 fibroblasts were grown in 96-well plates (Nunclon, Thermo Fischer Scientific, Rochester, NY). The growth medium consisted of Dulbecco's modified Eagle's medium (colorless) containing 2 mM L-glutamine, 100 mM pyruvate, 4.5 g/L glucose, 10% (v/v) fetal bovine serum, and 100 U/mL each of penicillin G and streptomycin. The concentration of the cell suspension was determined using a cell counter (Beckman Coulter ActDiff haematology analyzer, Fullerton, CA) and the suspension diluted with growth medium to give a cell concentration of 2×10^5 cells/mL. A 90 μL amount of this suspension was pipetted into each well and the plate incubated at 37 °C and 5% CO_2 (Sanyo CO_2 MCO-17 AIC incubator). After incubation for approximately 20 h, plant extracts and controls were added to the plate. Acetylsalicylic acid (Sigma A5376) was used as positive control. After addition of samples and controls the plate was incubated for a further 3 h. After 3 h incubation, 1 μL of calcium ionophore A21387 (calcimycin; Sigma C7522) solution (5 mM in DMSO) was added to each well to stimulate PGE_2 synthesis, and the plate was shaken gently on an orbital shaker for 20 s and incubated for a final 20 min. Immediately following incubation, the plate was centrifuged (Hettich Universal 16A centrifuge, Tuttlingen, Germany) for 3 min at 1000 RCF, and the supernatant was transferred to a clean 96-well plate, sealed, and frozen at −80 °C until the enzyme immunoassay was performed. The PGE_2 enzyme immunoassay was carried out using the thawed cell culture supernatant, which was diluted 500-fold with the buffer provided with the kit. A 50 μL amount of diluted sample was added to each well. A PGE_2 standard was run at eight concentrations in duplicate on each plate in all experiments. PGE_2 acetylcholine esterase tracer and PGE_2 monoclonal antibody were added (50 μL of each to each well), and the sealed plate was incubated at 4 °C for 18−20 h. Following incubation the plate was washed five times with the wash buffer provided in the kit and dried, and Ellman's reagent was added (200 μL per well). The plate was sealed, covered in aluminum foil, and placed on an orbital shaker (moderate speed) for 75−90 min, after which the absorbance at 405 nm of the developed plate was read in a Wallac Victor 2 plate reader (Perkin-Elmer, Waltham, MA). All samples, standards, and controls were assayed in triplicate. Raw data were obtained as concentrations of PGE_2 (pg/well). Standard curves for PGE_2 were plotted and R^2 values calculated to confirm linearity. Mean, standard deviation, and coefficient of variance were calculated for replicates of samples, standards, and controls. Inhibition of PGE_2 release by stimulated 3T3 cells was reported as percentage inhibition compared with stimulated cells treated with solvent controls.

Cytotoxicity Assays. Cytotoxicity in 3T3 murine fibroblasts, P388D1 murine lymphoblasts, Caco-2 human colonic adenocarcinoma, PC3 human prostate adenocarcinoma, HepG2 human hepatocyte carcinoma, and MCF7 human mammary adenocarcinoma (all cell lines originating from the American Type Culture Collection, ATCC) was assayed in 96-well plates using the ATPLite kit (PerkinElmer, Waltham, MA) with chlorambucil (Sigma C-0253) and curcumin (Sigma C-1386) as reference compounds. Cells were cultivated in the appropriate medium, and test and reference compounds were added at five different concentrations and incubated for 24 h (5% CO_2). All samples were assayed in triplicate. The ATPLite assay was carried out according to the manufacturer's instructions. Briefly, the kit components were equilibrated to room temperature. The lyophilized substrate solution was reconstituted with buffer, and this reagent was added to the plate containing the cells and incubated with test substances and standards. The plate was then shaken on an orbital microplate shaker (700 rpm) for 2 min before luminescence was measured on a Wallac Microbeta scintillation and luminescence counter (PerkinElmer, Waltham, MA). ATP was quantified using an ATP standard curve based on luminescence measurements of ATP standards. Half-maximal inhibitory concentration (IC_{50}) values were calculated using Excel 2003 (Microsoft Corporation, Redmond, WA) and GraphPad Prism 4 (San Diego, CA).

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Supporting Information Available: NMR data for compound **1** are available free of charge via the Internet at http://pubs.acs.org.

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