Cytotoxic Flavone Analogues of Vitexicarpin, a Constituent of the Leaves of Vitex negundo

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Cytotoxic Flavone Analogues of Vitexicarpin, a Constituent of the Leaves of *Vitex negundo*

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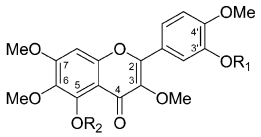
Bioassay-guided fractionation of the chloroform-soluble extract of the leaves of *Vitex negundo* led to the isolation of the known flavone vitexicarpin (**1**), which exhibited broad cytotoxicity in a human cancer cell line panel. In an attempt to increase the cytotoxic potency of **1**, a series of acylation reactions was performed on this compound to obtain its methylated (**2**), acetylated (**3**), and six new acylated (**4**−**9**) derivatives. Compound **9**, the previously unreported 5,3′-dihexanoyloxy-3,6,7,4′-tetramethoxyflavone, showed comparative cytotoxic potency to compound **1** and was selected for further evaluation. However, this compound was found to be inactive when evaluated in the in vivo hollow fiber assay with Lu1, KB, and LNCaP cells at the highest dose (40 mg/kg/body weight) tested, and in the in vivo P-388 leukemia model (135 mg/kg), using the ip administration route.

The genus *Vitex* of the family Verbenaceae is constituted by 250 species of small trees and shrubs which occur in tropical to temperate regions.[1] *Vitex negundo* L. is a small aromatic plant that is widely distributed in South Asia, the People's Republic of China, Indonesia, and the Philippines.[1] This plant has been used in traditional Indian medicine due to its anti-inflammatory properties for the treatment of rheumatoid arthritis.[1,2] Previous phytochemical studies on *V. negundo* have afforded several types of compounds, such as flavonoids,[2] iridoid glycosides,[3] lignans,[4] and sesquiterpenes.[5] As part of an ongoing collaborative search for novel antineoplastic agents of plant origin, cytotoxicity-based, bioassay-guided fractionation of the chloroform-soluble extract of the leaves of *Vitex negundo* L. (Verbenaceae), collected in Indonesia, led to the isolation of the known flavone vitexicarpin (**1**)[6−11] as the only active principle. In an attempt to increase the resultant cytotoxic potency of **1**, a series of acylation reactions was performed on compound **1** to afford derivatives **2**−**9**. In this paper, we describe the isolation of vitexicarpin (**1**), the synthesis of several analogues of **1** (**2**−**9**), the cytotoxicity determination of **1**−**9** in a human cancer cell line panel, and the in vivo evaluation of **9** in the hollow fiber assay[12−14] and in the P-388 leukemia model.[15]

A chloroform extract of the leaves of *V. negundo* showed a significant cytotoxic response against the Lu1 cell line with an IC_{50} value of 3.4 μg/mL. Bioassay-directed fractionation using this cell line led to the isolation of **1** as the active component. This result was in agreement with preliminary results obtained in our laboratory from LC-MS dereplication experiments[16] using the active fractions



	R_1	R_2
1	H	H
2	CH_3	CH_3
3	CH_3CO	CH_3CO
4	C_6H_5CO	H
5	C_6H_5CO	C_6H_5CO
6	CH_3CH_2CO	CH_3CH_2CO
7	$CH_3(CH_2)_2CO$	$CH_3(CH_2)_2CO$
8	$CH_2=CH(CH_2)_2CO$	$CH_2=CH(CH_2)_2CO$
9	$CH_3(CH_2)_4CO$	$CH_3(CH_2)_4CO$

F002 (Lu1, 5.9 μg/mL) and F003 (Lu1, 5.1 μg/mL), which indicated that the mass associated with cytotoxic activity against Lu1 cells corresponded to the molecular ion at 374 mass units. Compound **1** exhibited significant activity against the Lu1 (human lung) cancer cell line with an IC_{50} value of 0.8 μg/mL. In an attempt to increase the cytotoxic activity, and in order to obtain a preliminary notion of structure−cytotoxicity relationships, a series of acylation reactions (Figure 1 in Supporting Information) was performed with compound **1** to yield compounds **2**−**9**.

Vitexicarpin (**1**) was identified by comparison of its melting point and spectroscopic characteristics with literature values.[7,17−20] Compounds **4**−**9** are new derivatives of **1**, while compounds **2** and **3** have been reported previously in the literature.[17,20] The structures of compounds **4**−**9** were consistent with the spectroscopic values obtained in each case and were supported with $^1H-^1H$ COSY, HMQC, and HMBC experiments, and by HRMS analysis. The ^1H and ^{13}C NMR data for **4**−**8** are shown in Tables 1 and 2, respectively, of the Supporting Information.

The cytotoxic activities of compounds **1**−**9** were evaluated against a panel of human cancer cell lines (Table 1), according to established protocols.[21,22] Vitexicarpin (**1**) exhibited broad cytotoxic activity, except for the Col2 cell line. Permethylation and benzoylation of this compound to afford **2** and **4**−**5**, respectively, resulted in total inactivation

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Table 1. Cytotoxic Activity of Compounds **1**−**9**[a,b]

compound	Col2	hTERT-RPE1	HUVEC	KB	LNCaP	Lu1
1	12.7	0.2	0.6	0.2	0.5	0.8
2	>20	>20	>20	>20	>20	>20
3	15.9	0.4	0.1	0.2	0.1	0.7
4	>20	3.6	20	>20	>20	16.5
5	>20	4.3	>20	>20	>20	6.5
6	>20	0.5	6.5	0.6	0.4	1.0
7	11.7	0.6	>20	0.5	0.8	1.7
8	15.0	0.6	5.5	0.6	0.7	1.4
9	>20	0.4	11.1	0.5	0.5	0.7

[a] Results are expressed as ED_{50} values (μg/mL). [b] Key to cell lines used: Col2 = human colon cancer; hTERT-RPE1 = human telomerase reverse transcriptase-retinal pigment epithelial; HUVEC = human umbilical vein endothelial; KB= human oral epidermoid carcinoma; LNCaP = human hormone-dependent prostate cancer; Lu1 = human lung cancer.

or a significant reduction of cytotoxic activity. Compounds **3** and **6**−**9** showed cytotoxicity profiles similar to that of vitexicarpin (**1**) in the cell panel represented, with compounds **3** and **9** exhibiting minor increases in activity against the LNCaP and/or Lu1 cell lines.

Compounds **1**, **3**, **4**, and **6**−**9** were further evaluated for cytotoxicity in an 11-cell-line Oncology Diverse Cell Assay panel, representing a diverse group of human and murine tumor cells, according to previously established protocols.[23] Since these compounds showed good potency (mean IC_{50} value of <5.0 μM) and a high cell selectivity against the K562 human leukemia cell line (maximum/minimum IC_{50} ratio of >10) in the ODCA assay, they were considered as high-priority leads. Compound **9** was selected for in vivo hollow fiber and P-388 antileukemic evaluation rather than **1**, as it is a new compound.

Compound **9** inhibited the growth of KB, LNCaP, and Lu1 cells with ED_{50} values of 0.5, 0.5, and 0.7 μg/mL, respectively. Accordingly, compound **9** was evaluated with the in vivo hollow fiber system[12−14] using the KB, LNCaP, and Lu1 cell lines at doses of 10, 20, and 40 mg/kg. With LNCaP cells, compound **9** inhibited growth by 0−7.2% at the ip site and 0−2.4% at the sc site, while with KB cells, the compound was ineffective at the ip site and inhibited growth by 0−8.2% at the sc site. Finally, for Lu1 cells, this compound did not inhibit growth at the ip site, and only a modest response (0−2.1%) was observed at the sc site. No significant weight loss was observed in mice during this test procedure (Figure 2, Supporting Information).

Compound **9** was also evaluated in an in vivo mouse P-388 leukemia system under a standard protocol.[15] However, when administered intraperitoneally at 135 mg/kg/injection, this compound was deemed inactive (T/C value of 100%).

Experimental Section

General Experimental Procedures. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. UV spectra were measured on a Beckman DU-7 spectrometer. IR spectra were obtained with an ATI Mattson FT-IR spectrometer. NMR spectra were recorded with TMS as internal standard, using either a Bruker Avance-360 or a Bruker DRX-500 NMR spectrometer (360 or 500 MHz, respectively). HREIMS and LREIMS were recorded on a Finnigan MAT 95 mass spectrometer (70 eV), while HR-FABMS and LRFABMS were recorded on a VG70E-HF mass spectrometer. Column chromatography was carried out on silica gel 60 (Merck, Darmstadt, Germany; 63−200 and 230−400 mesh). Preparative TLC was performed on glass-backed TLC silica gel plates [2 mm thick (Scientific Adsorbents Inc., Atlanta, GA)]. Thin-layer chromatography (TLC) was performed on precoated 0.25 mm thick Merck silica gel F_{254} aluminum plates. Fractions were monitored by TLC with visualization under UV light (254 and 365 nm) and by dipping the plates into a solution of 10% (v/v) H_2SO_4 in EtOH.

Plant Material. Leaves of *V. negundo* were collected in July 1996 at Ujung Genteng, West Java, Indonesia (07°22′ S, 106°24′ E) by S.R. and J. J. Afriastini. A voucher specimen (J-050) has been deposited at the Field Museum of Natural History, Chicago, IL.

Extraction and Isolation. The air-dried, powdered leaves (788 g) were milled and extracted by maceration with MeOH (6 L × 3) in a percolator at room temperature. The MeOH solutions were combined, filtered, and evaporated under a vacuum. The dried MeOH extract (62.5 g) was dissolved in 1 L of a mixture of MeOH−H_2O (90:10) and then partitioned with hexane (500 mL × 6) to afford a dried hexane extract (20.1 g). The aqueous MeOH solution was then partitioned with $CHCl_3$ (500 mL × 5). The combined organic layer was washed using 1% saline solution and concentrated under vacuum to yield 18.7 g of $CHCl_3$ extract, which showed significant cytotoxic activity against the Lu1 cell line with an IC_{50} value of 3.4 μg/mL.

The $CHCl_3$-soluble extract (18.4 g) was subjected to fractionation using an open column with 540 g of silica gel (63−200 mesh) and eluted with a gradient of increasing polarity with petroleum ether−$CHCl_3$−MeOH. Ten fractions (F1−F10) were obtained and monitored using Lu1 cells. An orange, amorphous solid was precipitated from a hexane−$CHCl_3$ solution of the active fractions F2 (Lu1, 5.9 μg/mL) and F3 (Lu1, 5.1 μg/mL). This solid was subjected to several purification steps by crystallization in hexane and MeOH to produce orange crystals corresponding to the structure of compound **1** (272 mg), which showed a strong cytotoxic response (ED_{50}: 0.8 μg/mL) against the Lu1 cell line. No other cytotoxic compound was isolated from the $CHCl_3$-soluble extract in the present study.

Vitexicarpin (1): orange crystals ($CHCl_3$−MeOH); mp 188−190 °C (lit.[7] 188−189 °C). Compound **1** exhibited comparable spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, and EIMS) data to published values.[17−20]

3,5,6,7,3′,4′-Hexamethoxyflavone (2). Permethylation of 5 mg of vitexicarpin (**1**) was achieved using the Aldrich Diazald kit to generate CH_2N_2. Compound **2** (4.9 mg; 90.7% yield) was obtained as a pale yellow, amorphous solid, mp 179−180 °C. Compound **2** exhibited comparable spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, and EIMS) data to published values.[17]

Acylation of Vitexicarpin (1). Five milligrams of compound **1** was dissolved with pyridine (0.3 mL) and treated with the respective anhydride (0.5 mL) to yield compounds **3**−**9**. Each mixture was left to stand at room temperature overnight. After removal of the reagent under a dried-air stream, the residue was partitioned between $CHCl_3$ (5 mL × 3) and water (5 mL). The organic layer was concentrated to afford a pale yellow, amorphous solid in each case (Figure S1, Supporting Information).

5,3′-Diacetoxy-3,6,7,4′-tetramethoxyflavone (3): mp 140−142 °C. Compound **3** exhibited comparable spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, and EIMS) data to published values.[20]

3′-Benzoyloxy-5-hydroxy-3,6,7,4′-tetramethoxyflavone (4): mp 210−211 °C; UV ($CHCl_3$) λ_{max} (log ϵ) 333 (4.36), 276 (4.33), 241 (4.41) nm; IR (dried film) ν_{max} 2924, 2365, 2333, 1742, 1655, 1458, 1364, 1222, 812, 710 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables S1 and S2; LREIMS *m*/*z* 478 (47) $[M]^+$, 463 (12), 435 (1) 105 (100); HREIMS *m*/*z* 478.1264 ($C_{26}H_{22}O_9$, calcd 478.1264).

5,3′-Dibenzoyloxy-3,6,7,4′-tetramethoxyflavone (5): mp 220−222 °C; UV ($CHCl_3$) λ_{max} (log ϵ) 322 (4.34), 282 (sh, 3.93), 262 (4.18), 241 (4.53) nm; IR (dried film) ν_{max} 2916, 2848, 1742, 1611, 707 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables S1 and S2; LREIMS *m*/*z* 582 (25), 477 (28), 105 (100); HREIMS *m*/*z* 582.1493 ($C_{33}H_{26}O_{10}$, calcd 582.1526).

5,3′-Dipropanoyloxy-3,6,7,4′-tetramethoxyflavone (6): mp 131−132 °C; UV ($CHCl_3$) λ_{max} (log ϵ) 320 (4.07), 265 (4.15), 241 (4.04) nm; IR (dried film) ν_{max} 2938, 2848, 1764, 1623, 1512, 1464, 1358, 1276 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables

S1 and S2; LRFABMS *m*/*z* 487 $[M + H]^+$; HRFABMS *m*/*z* 509.1437 ($C_{25}H_{26}O_{10}Na$, calcd 509.1423).

5,3′-Dibutanoyloxy-3,6,7,4′-tetramethoxyflavone (7): mp 108−109 °C; UV ($CHCl_3$) λ_{max} (log ϵ) 321 (4.25), 264 (4.23), 243 (4.08) nm; IR (dried film) ν_{max} 2961, 2338, 1762, 1623, 1467, 1360, 1277 cm^{-1}; ^1H and ^{13}C NMR data, see Tables S1 and S2; LRFABMS *m*/*z* 515 $[M + 1]^+$; HRFABMS *m*/*z* 515.1912 ($C_{27}H_{31}O_{10}$, calcd 515.1917).

5,3′-Dipent-4-enoyloxy-3,6,7,4′-tetramethoxyflavone (8): mp 155−157 °C; UV (MeOH) λ_{max} (log ϵ) 327 (4.70), 261 (4.60), 234 (sh, 4.60) nm; IR (dried film) ν_{max} 1764, 1623, 1462, 1362, 1276 cm^{-1}; ^1H and ^{13}C NMR data, see Tables S1 and S2; LRFABMS *m*/*z* 539 $[M + 1]^+$; HRFABMS *m*/*z* 539.1908 ($C_{29}H_{31}O_{10}$, calcd 539.1917).

5,3′-Dihexanoyloxy-3,6,7,4′-tetramethoxyflavone (9): mp 100−101 °C; UV (MeOH) λ_{max} (log ϵ) 324 (4.30), 260 (4.09), 229 (4.22) nm; IR (dried film) ν_{max} 2935, 2355, 2338, 1764, 1623, 1458, 1358, 1275 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 8.02 (1H, dd, J = 8.8, 2.2 Hz, H-6′), 7.77 (1H, d, J = 2 Hz, H-2′), 7.06 (1H, d, J = 8.8 Hz, H-5′), 6.85 (1H, s, H-8), 2.82 (2H, t, J = 7.5 Hz, H-2‴), 2.63 (2H, t, J = 7.5 Hz, H-2″), 1.89 (2H, t, J = 7.3 Hz, H-3″), 1.85 (2H, t, J = 7.3 Hz, H-3‴), 1.44 (4H, m, H-4″, H-4‴), 1.40 (4H, m, H-5″, H-5‴), 0.95 (6H, m, H-6″, H-6‴); ^{13}C NMR ($CDCl_3$, 125.78 MHz) δ 172.1 (C, C-1″, C-1‴), 171.8 (C, C-4), 157.6 (C, C-7), 153.0 (C, C-2), 152.9 (C, C-4′), 142.1 (C, C-3), 140.9 (C, C-3′), 139.8 (C, C-6), 127.5 (CH, C-6′), 123.3 (C, C-1′), 122.8 (CH, C-2′), 112.1 (CH, C-5′), 111.9 (C, C-10), 97.9 (CH, C-8), 61.5 (CH_3, OCH_3-6), 59.9 (CH_3, OCH_3-3), 56.4 (CH_3, OCH_3-7), 55.9 (CH_3, OCH_3-4′), 33.9 (CH_2, C-2″), 34.2 (CH_2, C-2‴), 31.3 (CH_2, C-4‴), 31.2 (CH_2, C-4″), 24.6 (CH_2, C-3‴), 24.3 (CH_2, C-3″), 22.4 (CH_2, C-5″), 22.3 (CH_2, C-5‴), 14.0 (CH_3, C-6″, 6‴); LRFABMS *m*/*z* 571 $[M + 1]^+$; HRFABMS *m*/*z* 593.2358 ($C_{31}H_{38}O_{10}Na$, calcd 593.2363).

Bioassay Evaluation Procedures. The cytotoxic activity of compounds **1**−**9** was evaluated against a panel of human cancer cell lines (Table 1), according to established protocols.[22,23] Compounds **1**, **3**, **4**, and **6**−**9** were further evaluated in a 11-cell-line Oncology Diverse Cell Assay (ODCA),[23] using a MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfenyl)-*2H*-tetrazolium, inner salt] assay.

In Vivo Evaluation of Compound 9. Compound **9** was assessed for its biological potential in the in vivo hollow fiber[12−14] (25 mg quantity used) and P-388 leukemia[15] (200 mg quantity used) models as described.

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Supporting Information Available: The ^1H and ^{13}C NMR data for the new analogues **4**−**8** of vitexicarpin (**1**) (Tables S1 and S2), a summary of acylation reactions for compound **1** (Figure S1), and the results from the in vivo hollow fiber study performed with compound **9** (Figure S2). This information is available free of charge via the Internet at http://pubs.acs.org.

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