Isolation and Structure of Palstatin from the Amazon Tree *Hymeneae palustris*[1]

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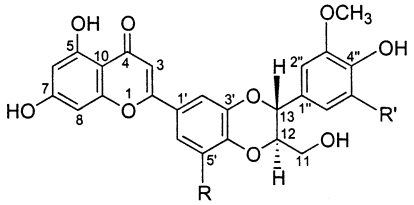
Bioassay (P388 lymphocytic leukemia cell line and human cancer cell lines) guided separation of an extract prepared from the leaves of *Hymenaea palustris* Ducké led to the isolation of six cancer cell growth inhibitory flavonoids (**1**−**6**). The structures were elucidated by HRMS and 1D and 2D NMR spectral analysis. The new flavonolignan **1** designated palstatin proved to be a methoxy structural modification of 5′-methoxyhydnocarpin-D (**2**). Flavones **1**−**4** inhibited growth of the pathogenic bacteria *Enterococcus faecalis* and/or *Neisseria gonorrhoeae*.

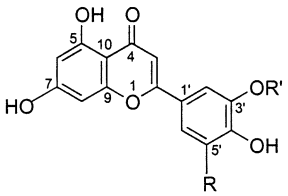
Tropical trees contained in the Leguminosae genus *Hymenaea* are primarily found in the Amazon Basin, but range into Central America and East Africa.[2a−c] Approximately 25 *Hymenaea* species from the American continent have been described. *Hymenaea* trees, such as *H. verrucosa*[2c] found in East Africa, provide commercial resins known as Zanzibar and Madagascar copal used in the manufacture of varnishes and shellacs.[2c] The tree resin exudates contain substantial amounts of diterpene carboxylic acids.[2b,c,3]

A few examples of biological activities for *Hymenaea* extracts have been reported. The Brazilian *H. courbaril* L. provided an extract with high 5-lipoxygenase inhibitory activity,[4] and the leaf resin exhibited toxic and feeding-deterrent properties, which made it effective as an herbivore defense.[5] The extract of *H. courbaril* has also been used in the cosmetics industry owing to the polycatechin it contains, which has moisturizing and skin-lightening effects.[6,7] An extract of the bark of *H. martiana* Hayne Arzeik has shown differential antagonistic effects and agonist-induced contractions of the isolated rat uterus and pig ileum.[8]

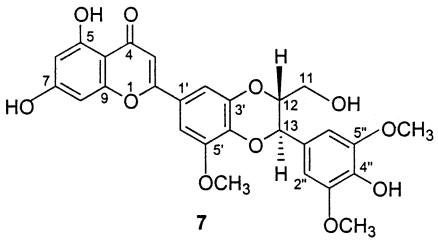
Specimens of the Peruvian tree *Hymenaea palustris* Ducké (Leguminosae) were collected 26 years ago as part of the U.S. National Cancer Institute's exploratory anticancer drug lead development research. Evaluation of an aqueous ethanol extract of the leaf against the murine in vivo P388 lymphocytic leukemia provided a 43%−59% increase in survival at 90−150 mg/kg. A 1976 scale-up recollection of the leaf allowed an investigation focused on isolating the antineoplastic constituent(s). Due to termination of the NCI in vivo P388 leukemia for bioassay purposes (when the present research was initially underway in 1981), combined with only a marginal response of *H. palustris* fractions to the P388 in vitro cell line, further progress had to await our Institute's implementation of human cancer cell line screening procedures, which enabled us to isolate six human cancer cell-growth inhibitory flavonoids (**1**−**6**) from *H. palustris* and determine their antimicrobial activities. Among the six flavonoids, five have been previously isolated from other sources, but one flavonolignan proved to be a new cancer cell growth inhibitor and was designated palstatin (**1**). These are the first constituents isolated from *H. palustris*.



1, R = R' = OCH_3 (palstatin)
2, R= OCH_3; R' = H
3, R = R' = H



4, R = R' = H
5, R = H, R' = CH_3
6, R = OCH_3, R' = CH_3



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Results and Discussion

A CH_2Cl_2−MeOH (1:1) extract of *Hymenaea palustris* leaves (22.1 kg) was successively partitioned between MeOH−H_2O (9:1 → 1:1) and hexane followed by CH_2Cl_2. Bioassay-guided separation of the CH_2Cl_2 phase using a series of gel permeation and partition separations on Sephadex LH-20 followed by final purification by HPLC or by recrystallization afforded the new flavonolignan palstatin (**1**, 2.6×10^{-5} yield), the known flavonolignans **2** (1.6×10^{-4} yield) and **3** (1.5×10^{-5} yield), and the previously known flavones **4** (3.7×10^{-5} yield), **5** (1.3×10^{-5} yield), and **6** (7.6×10^{-5} yield).

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Table 1. ^{1}H and ^{13}C NMR Assignments for Palstatin (**1**) in DMSO[a]

position	δ^1H	^{1}H−^{1}H COSY	δ^{13}C	HMBC[b]
2			162.9	H-3, H-2′, H-6′
3	6.95 s (1H)		104.1	
4			181.8	H-3, H-8 (w)
5			161.4	H-6, 5-OH
6	6.16 d (1H, 2.5)		99.0	H-8
7			164.6	H-6, H-8
8	6.49 d (1H, 2.5)		94.2	H-6
9			157.4	H-8
10			103.6	H-6, H-8, H-3, 5-OH
11a	3.36 dd (1H, 11, 4)	11b, 12	59.9	
11b	3.61 d (1H, 11)	11a		
12	4.25 dd (1H, m)	11b, 13	78.3	H-13
13	4.93 d (1H, 8)	12	76.0	H-12, H-2″, 6″
1′			122.3	H-3, H-2′, H-6′
2′	7.31 d (1H, 3.5)		108.1	H-6′
3′			144.2	H-2′
4′			136.6	H-2′, H-6′
5′			149.0	5′-OMe, H-6′
6′	7.26 d (1H, 3.5)		102.8	H-2′
5′-OMe	3.91 s (3H)		56.0	
1″			126.1	H-13, H-2″, 6″
2″, 6″	6.73 s (2H)		105.4	H-13, H-6″, 2″
3″, 5″			147.9	3″, 5″-OMe, H-2″, 6″
4″			136.0	H-2″, 6″
3″, 5″-OMe	3.76 s (6H)		56.1	
5-OH	12.89 s (1H)			

[a] Measured at 500 MHz. [b] w = weak.

The molecular formula of palstatin (**1**) was assigned as $C_{27}H_{24}O_{11}$ on the basis of high-resolution FAB mass spectroscopy. The new compound exhibited UV maxima at 269 and 341 nm, which were characteristic of a flavonoid.[9] The ^{1}H NMR spectrum showed signals (H-3, 6, 8, 2′, and 6′ in Table 1) typical of a tricin-like flavone skeleton. The ^{13}C NMR (DEPT, Table 1) spectra presented nine carbon signals corresponding to a C_6−C_3 phenylpropane unit, and the frequencies were characteristic of a flavone skeleton. The deshielded doublet (δ 4.93) observed in the ^{1}H NMR spectrum suggested a benzylic methyne substituted by oxygen, and the trans-coupling (J = 8 Hz) of the doublet indicated the presence of a trans-substituted 1,4-dioxane ring.[10] Thus, **1** was preliminarily determined to be a flavonolignan, a class of compound whose biosynthesis is presumed to follow radical coupling of a catechol with a coniferyl alcohol type unit.[11]

The ^{1}H−^{1}H COSY (Table 1) revealed the coupling patterns of the oxygenated methyne and methene protons of H-11, H-12, and H-13. The HMQC and HMBC (Table 1) 2D NMR spectral analyses confirmed that the 1,4-dioxane ring formed between the flavone 2-phenyl ring and coniferyl unit was situated at C-2′ and C-3′, the two phenol hydroxyl groups at C-5 and C-7, the isolated phenol at C-4″, and the three methoxy groups at C-5′, C-3″, and C-5″, respectively. The regioisomeric orientation (cf. **1** vs **7**) of the coniferyl unit was not easily deduced on the basis of the HMBC spectrum and other available 1D and 2D NMR data. As we were unable to induce the amorphous palstatin to yield crystals suitable for X-ray crystal structure determination, we have for the present relied on the close spectral relationship with the known flavonolignan **2** we also isolated from *H. palustris*. Presumably an analogous *H. palustris* biosynthesis led to both flavones **1** and **2** combined with correlation patterns that appear in the HMBC spectra of both. The structure of palstatin (**1**) was thus deduced to be a 5″-methoxy derivative of 5′-methoxyhydnocarpin-D.[12]

Table 2. Murine P388 Lymphocytic Leukemia Cell Line and Human Cancer Cell Line Inhibition Values (GI_{50} expressed in μg/mL) for Flavonoids **1**−**6**[a]

cancer cell line[b]	flavonoids					
	1	**2**	**3**	**4**	**5**	**6**
P388	8.5	2.7	9.5	>10	4.0	>10
BXPC-3	3.2	1.8	1.5	>10	2.6	7.5
MCF-7	3.6	2.4	2.7	9.4	3.1	7.7
SF268	>10	2.2	2.6	9.0	1.7	3.0
NCI-H460	5.9	2.0	2.3	3.8	2.1	6.5
KM20L2	>10	1.9	2.0	>10	2.5	>10
DU-145	>10	1.8	1.9	4.4	1.1	4.6

[a] In DMSO. [b] Cancer type: P388 (lymphocytic leukemia); BXPC-3 (pancreas adenocarcinoma); MCF-7 (breast adenocarcinoma); SF268 (CNS glioblastoma); NCI-H460 (lung large cell); KM20L2 (colon adenocarcinoma); DU-145 (prostate carcinoma).

Flavonolignan **2** was also isolated as a pale yellow amorphous powder with molecular formula $C_{26}H_{22}O_{10}$ on the basis of high-resolution APCI$^+$ mass spectroscopy. The UV maxima at 270 and 341 nm suggested a flavonoid.[9] By interpretation of its ^{1}H and ^{13}C NMR, ^{1}H−^{1}H COSY, HMQC, and HMBC spectra followed by comparison with published ^{1}H and ^{13}C NMR data,[12] the structure was found to be 5′-methoxyhydnocarpin-D.[12] Also we found that the previously[12] reported NMR assignments for C-2 and C-7 required reversal because of the strong correlations from H-2′, H-6′, and H-3 to C-2, and from H-8 and H-6 to C-7 observed in its HMBC spectrum. Parallel spectral techniques were employed to characterize the remaining yellow flavonoids as hydnocarpin-D (**3**),[11a] also with correction of the previous[11a] C-2 and C-7 NMR assignments, luteolin (**4**)[13] and chrysoeriol (**5**),[14] with a similar correction of assignments for C-5 and C-9,[15] and tricin (**6**).[16,17]

Numerous biological activities of flavonoids **2**−**6** have been reported. For example, tricin (**6**) has been evaluated with respect to antimicrobial[18] and cancer cell line[19] activities; chrysoeriol (**5**) and luteolin (**4**) have been tested for cancer cell cytotoxic,[20] antioxidant,[21] antiinflammatory,[22] and antimicrobial[23,24] activities; hydnocarpin-D (**3**) has been tested for hypolipidemic, cytotoxic, and antiinflammatory properties;[25] and 5′-methoxyhardnocarpin-D (**2**) has been studied as a potent inhibitor of the *Staphylococcus aureus* multidrug-resistant efflux pump.[11] In the present investigation, flavonoids **1**−**6** were first examined using the murine P388 lymphocytic leukemia cell line and a selection of human cancer cell lines. All were found, as expected from the isolation bioassay results, to exhibit cancer cell growth inhibitory activities ranging from significant to marginal (Table 2). Earlier[20a] we had observed an analogous result by employing the P388 leukemia cell line to isolate luteolin (**4**) as a significant growth inhibitory component of a Republic of Mauritius *Terminalia* (Combretaceae) tree.

In broth microdilution susceptibility assays,[26,27] flavones **1**−**4** inhibited growth of the Gram-negative pathogen *Neisseria gonorrhoeae* (minimum inhibitory concentrations: 1, 0.625, 0.5, and 16−32 μg/mL, respectively). Flavones **2** and **4** also inhibited growth of the Gram-positive opportunist *Enterococcus faecalis* with MICs of 32 and 64 μg/mL, respectively. In these assays, flavones **5** and **6** showed no inhibition of *Neisseria gonorrhoeae* or *Entero-*

coccus faecalis. None of the flavones **1**−**6** inhibited the growth of *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Micrococcus luteus*, *Enterobacter cloacae*, *Escherichia coli*, *Stenotrophomonas maltophilia*, *Cryptococcus neoformans*, or *Candida albicans* (up to 64 μg/mL).

Experimental Section

General Experimental Procedures. All chromatographic solvents were redistilled. Sephadex LH-20 used for partition column chromatography was obtained from Pharmacia Fine Chemicals AB. Analytical HPLC was conducted with a Hewlett-Packard Model 1050 HPLC coupled with a Hewlett-Packard diode-array detector. Semipreparative HPLC was performed with a Gilson Model 805 HPLC coupled with a Gilson Model 117 UV detector. The optical rotation measurement for palstatin was recorded with a Perkin-Elmer 241 polarimeter. Melting points were measured using an Olympus electrothermal melting point apparatus and were uncorrected. UV spectra were collected with a Perkin-Elmer Lambda 3B UV/vis spectrometer, and NMR spectra were recorded with a Varian XL-300 or a Varian UNITY INOVA-500 spectrometer with tetramethylsilane (TMS) as an internal reference. High-resolution mass spectra were obtained using a JEOL LCMate magnetic sector instrument either in the FAB mode with a glycerol matrix or by APCI with a poly(ethylene glycol) reference.

Plant Collection. *Hymenaea palustris* Ducké (Leguminosae) was collected in Peru in 1974−1976. Both the original and 1976 re-collection of the *H. palustris* leaves were obtained during the joint U.S. National Cancer Institute/U.S. Department of Agriculture research endeavors under the direction of Drs. John D. Douros and Matthew I. Suffness in the NCI, and Robert E. Purdue and James A. Duke in the USDA. A voucher specimen of *H. palustris* was deposited in the Medicinal Plant Resources Laboratory of the USDA, Beltsville, MD.

Extraction and Initial Separation of *H. palustris*. A 22.1 kg sample of the 1976 re-collection (103.5 kg) of *H. palustris* leaves was twice extracted (initial for 10 days and repeat for 5 days) with 1:1 CH_2Cl_2−MeOH (2 × 208 L). After each extraction, 30% H_2O by volume was added to separate the CH_2Cl_2 phase. Solvent was removed in vacuo to provide from the first CH_2Cl_2 fraction 627 g (P388 ED_{50} 39 μg/mL) and from the second fraction 154 g (P388 ED_{50} 54 μg/mL). The fractions were combined and partitioned between solvent systems composed of MeOH−H_2O (9:1 → 1:1), hexane (with 9:1), and CH_2Cl_2 (for the 1:1) to afford 187 g of a CH_2Cl_2 layer with somewhat improved activity (P388 ED_{50} 27 μg/mL). The solvent partitioning sequence was a modification of the original procedure of Bligh and Dyer.[28]

Isolation. The CH_2Cl_2 fraction (187 g) was passed through a Sephadex LH-20 column, using CH_3OH as eluent. Both P388 and human cancer cell line active fractions (a, b, c, d, and e) were obtained. Fractions a, b, c, and d were combined and rechromatographed on a Sephadex LH-20 column, using hexane−CH_3OH−2-propanol (8:1:1) as eluent, to provide three active fractions. All were combined and dissolved in 20 mL of *n*-hexane−toluene−acetone−CH_3OH (1:4:3:1). After removing some sparingly soluble material, the solution was rechromatographed on a Sephadex LH-20 column, using *n*-hexane−toluene−acetone−CH_3OH (1:4:3:1) as eluent, and three active fractions were obtained. The three active fractions together with the sparingly soluble material were combined and rechromatographed on a Sephadex LH-20 column, using hexane−acetone−CH_2Cl_2 (4:3:3) as eluent. That allowed the inhibitory activity to be concentrated into one active fraction, which was dissolved in *n*-hexane−toluene−acetone−CH_3OH (1:4:1:1) excluding less soluble material. The latter was collected and recrystallized (3×) from CH_3OH to afford pure flavonoid (**6**) as yellow needles (16.8 mg).

The CH_3OH filtrates from this recrystallization sequence were combined and chromatographed on a Sephadex LH-20 column, using toluene−ethyl acetate−CH_3OH (4:1:1) as eluent. One active fraction was obtained and further separated by HPLC, using a SYNERGI 4μ polar RP80A semipreparative column and a gradient mobile phase (30−39% CH_3CN in H_2O for 40 min, then 100% CH_3CN for a further 15 min). Peaks were monitored at 230 nm; flow rate was 2.8 mL/min. Pure flavonoid **5** (a yellow amorphous powder, 2.8 mg) was obtained. When the original fraction e was rechromatographed on a Sephadex LH-20 column, using CH_3OH−dichloromethane (3:2) as the eluent, three active fractions (f, g, and h) were obtained. The first two (f and g) were combined and partially dissolved in hexane−2-propanol−CH_3OH (8:1:1). The less soluble part was removed and partially dissolved in hot CH_3OH. The remaining less soluble material was removed and recrystallized from a large volume of CH_3OH. A yellow amorphous solid precipitated from the solution. The precipitate was collected and separated by semipreparative HPLC, using a SYNERGI 4μ polar semipreparative column and an isocratic gradient mobile phase (38% CH_3CN in H_2O for 40 min, then 100% CH_3CN for a further 20 min). The peaks were monitored at 230 nm; flow rate was 2.8 mL/min. Both palstatin (**1**, 5.8 mg) and flavonoid **2** (pale yellow amorphous powder, 35.4 mg) were obtained. The mother solution was chromatographed on a Sephadex LH-20 column, using toluene−ethyl acetate−CH_3OH (3:1:1) as eluent. One fraction was obtained and further separated by semipreparative HPLC, using the same column and conditions as summarized above for isolating flavonoids **1** and **2**. That led to a pure specimen of flavonoid **3** as a pale yellow amorphous solid (3.4 mg).

The fraction h was rechromatographed on a Sephadex column, using the *n*-hexane−toluene−acetone−CH_3OH (1:4:1:1) system as eluent. Three active fractions were obtained and combined. Partial solution in hexane−dichloromethane−acetone (4:3:3) left a solid residue that was recrystallized (2×) from CH_3OH to give pure flavonoid **4** as a pale yellow amorphous solid (8.2 mg).

Palstatin (1): pale yellow amorphous powder from MeOH; mp 238−239 °C; $[\alpha]^{24}_D$ 0 °C; UV λ_{max} (CH_3OH) 269 (log ϵ 4.0), 341 (log ϵ 4.1) nm; ^1H and ^{13}C NMR (see Table 1); HRFABMS *m/z* 525.1383 $[M + H]^+$ (calcd for $C_{27}H_{25}O_{11}$, 525.1397).

5′-Methoxyhydnocarpin D (2):[12] pale yellow amorphous powder from MeOH; mp 224−226 °C; HRMS (APCI pos.) *m/z* 513.1396 $[M + H + H_2O]^+$ (calcd for $C_{26}H_{25}O_{11}$, 513.1397); HRFABMS *m/z* 495.1252 $[M + H]^+$ (calcd for $C_{26}H_{23}O_{10}$, 495.1291).

Hydnocarpin D (3):[11] pale yellow amorphous powder from MeOH; mp 245 °C (dec); HRMS (APCI pos.) *m/z* 483.11998 $[M + H + H_2O]^+$ (calcd for $C_{25}H_{23}O_{10}$, 483.1291); HRFABMS *m/z* 465.1165 $[M + H]^+$ (calcd for $C_{25}H_{21}O_9$, 465.1185).

Luteolin (4):[13] yellow amorphous powder from MeOH; mp >300 °C; HRMS (APCI pos.) *m/z* 287.0565 $[M + H]^+$; HRFABMS *m/z* 287.0554 $[M + H]^+$ (calcd for $C_{15}H_{11}O_6$, 287.0556).

Chrysoeriol (5):[14] yellow amorphous powder from MeOH; mp >300 °C; HRMS (APCI pos.) *m/z* 301.0710 $[M + H]^+$; HRFABMS *m/z* 301.0702 $[M + H]^+$ (calcd for $C_{16}H_{13}O_6$, 301.0712).

Tricin (6):[16,17] yellow needles from methanol; mp 284 °C (dec); HRMS (APCI pos.) *m/z* 331.0833 $[M + H]^+$ (calcd for $C_{17}H_{15}O_7$, 301.0818).

Cancer Cell Line Procedure. The National Cancer Institute's standard sulforhodamine B assay was used to assess inhibition of human cancer cell growth as previously described.[29] In summary, cells in 5% fetal bovine serum/RPMI-1640 medium were inoculated in 96-well plates and incubated for 24 h. Serial dilutions of the compounds were then added. After 48 h, the plates were fixed with trichloroacetic acid, washed, stained with sulforhodamine B, and read with an automated microplate reader. Growth inhibition of 50% (GI_{50}, drug concentration causing a 50% reduction in the net protein increase) was calculated from optical density data with Immunosoft software. The murine P388 lymphocytic leukemia cell line results were obtained using 10% horse serum/Fisher medium, with incubation for 24 h. Serial dilutions of the compounds were added, and after 48 h, cell growth inhibition (ED_{50}) was calculated using a Z1 Coulter particle counter.

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