Isolation of Cytotoxic Metabolites from Targeted Peruvian Amazonian Medicinal Plants

José C. Aponte,[†] Abraham J. Vaisberg,[‡#] Rosario Rojas,[§] Luz Caviedes,[‡] Walter H. Lewis,[⊥] Gerardo Lamas,[||] César Sarasara,[▽] Robert H. Gilman,[‡○] and Gerald B. Hammond*[,†#]

Department of Chemistry, University of Louisville, Louisville, Kentucky 40292, Departamento de Microbiología y Laboratorios de Investigación y Desarrollo, Universidad Peruana Cayetano Heredia, Lima, Perú, Departamento de Ciencias Farmacéuticas, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia, Lima, Perú, Department of Biology, Washington University, St. Louis, Missouri 63130, Departamento de Entomología, Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Lima, Perú, Confederacion de Nacionalidades Amazónicas del Perú (CONAP), Lima, Perú, and Department of International Health, Johns Hopkins Uiversity School of Public Health, Baltimore, Maryland 21205

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The antiproliferative bioassay-guided fractionation of five Peruvian plants, *Doliocarpus dentatus*, *Picramnia sellowii*, *Strychnos mitscherlichii, Iryanthera juruensis*, and *Croton alnifolius*, led to the isolation and identification of their different major cytotoxic constituents, betulinic acid (**1**), nataloe-emodin (**2**), bisnordihydrotoxyferine (**4**), 2′,4′-dihydroxy-6′-methoxy-3,4-methylenedioxydihydrochalcone (**5**), and 2′,4′-dihydroxy-4,6′-dimethoxydihydrochalcone (**6**) and 12-*O*-tetradecanoylphorbol-13-acetate (**7**), respectively. Eight human tumor cell lines and two nontumorigenic cell lines were used in this investigation. Their in vitro activity against *Mycobacterium tuberculosis* is also reported.

Plant-based cancer therapies used in traditional medicine are more often than not viewed with skepticism because cancer is likely to be poorly defined in terms of folklore and traditional medicine. Nevertheless, it is significant that over 60% of currently used anticancer agents are derived in one way or another from natural sources.[1] Today, almost 80% of people in developing countries still rely on traditional folk medicine as their primary source of medicinal care.[2] In the mid 1990s, as a result of our participation in an International Cooperative Biodiversity Group (ICBG) program,[3] we began ethnobotanical research in the Peruvian upper Amazon basin and eastern Andes Mountain foothills among the Aguaruna community. A large number of the species chosen, prescreened for human use by the Aguaruna themselves, provided higher frequencies of bioactive secondary metabolites than those found in the flora as a whole.[4] By September 2006, 3591 collections with material for extraction had been completed. This plant inventory represents more than 127 families and 1000 taxa, with the majority being angiosperms. Over 80% of the plant species in this inventory were collected on the basis of their medicinal use, toxicity, or other physiological activity recognized and kept as part of the oral tradition of the Aguaruna community.

To date, cytotoxic activities of 2553 crude plant extracts have been assayed using a murine fibroblast nontransformed cell line as control and eight human cancerous cell lines. As illustrated in Figure S1 (Supporting Information), extracts from 304 species have shown significant cytotoxicity, with 50 species exhibiting high cytotoxicity (HC, 15.4%, $GI_{50} < 4$ μg/mL) and 109 moderate cytotoxicity (MC, 38.3%, GI_{50} between 16 and 4 μg/mL). Moreover, 24 species exhibited high differential cytotoxicity (HDC, 8.2%), 28 moderate differential cytotoxicity (MDC, 9.2%), and 93 low differential cytotoxicity (LDC, 28.9%). We characterize a HDC when the GI_{50} for the nontransformed 3T3 cells is at least 60 times higher than the corresponding values for the tumor cell lines. For the MDC and LDC, the GI_{50} values for the 3T3 cells are 25 and 10 times higher than those for the tumor cell lines, respectively.

In addition, using the tetrazolium microplate assay (TEMA), a group of 977 ethanol extracts from Peruvian plants were screened for antimycobacterial activity in vitro against the sensitive *Mycobacterium tuberculosis* (MTB) H37Rv strain.[5] Fifty-nine (6.04%) extracts showed MIC values ≤ 100 μg/mL.

In this report we present the results of cytotoxicity studies conducted on five Peruvian plants, *Doliocarpus dentatus* (Aublet) Standley (Dilleniaceae), *Picramnia sellowii* Planch (Simaroubaceae), *Strychnos mitscherlichii* M.R. Schomb (Loganiaceae), *Iryanthera juruensis* Warb (Myristicaceae), and *Croton alnifolius* Lam (Euphorbiaceae). To the best of our knowledge, this is the first laboratory study on *S. mitscherlichii* and *C. alnifolius*. Herein, we also present the results of the anti-MTB activity of the isolated metabolites responsible for the cytotoxic activity of each plant, except for the ones found in *C. alnifolius*.

Results and Discussion

Doliocarpus dentatus is widely distributed in the low tropical rainforests of Mesoamerica, from southern Mexico to northern Perú, western Brazil, and northern Bolivia. In northwestern Perú, the vine water of *D. dentatus*, known in the Aguaruna community as "waúna", is used to treat aftereffects of malaria. A previous study of *D. dentatus* identified antileishmanial principles.[6] In the present work, the bioassay-guided fractionation of the diethyl ether extract of *D. dentatus* led to the isolation in high yield (up to 53%) of betulinic acid (**1**) as the cytotoxic active metabolite. The highest cytotoxicity for **1** was found against the K562 cell line ($GI_{50} =$ 10.8 μg/mL, Table 1). Compound **1** did not show anti-MTB activity at 25 μg/mL. The structure of this known compound was determined on the basis of comparison of its spectroscopic data with literature information.[7]

Picramnia sellowii, a member of the new Picramniaceae family,[8] is widely distributed in the tropical rainforests of South America. In northwestern Perú, the leaves of *P. sellowii*, known in the Aguaruna community as "yamakai", are crushed and rubbed on the skin after bathing to eliminate mites, while the crushed bark and juice are used to dye red materials and white clothes. Triterpenoids and anthraquinonine derivatives have been isolated previously from *P. sellowii*.[9] The bioassay-guided fractionation of the ethanol extract led to the isolation and identification of nataloe-

* To whom correspondence should be addressed. Tel: (+1)502-852-5998. Fax: (+1)502-852-3899. E-mail: gb.hammond@louisville.edu.
[†] University of Louisville.
[‡] Departamento de Microbiología y Laboratorios de Investigación y Desarrollo, Facultad de Ciencias y Filosofía, Universidad Peruana Cayetano Heredia.
[§] Departamento de Ciencias Farmacéuticas, Universidad Peruana Cayetano Heredia.
[⊥] Washington University, St. Louis.
[||] Universidad Nacional Mayor de San Marcos.
[▽] Confederacion de Nacionalidades Amazónicas del Perú.
[○] Johns Hopkins University School of Public Health.
[#] A.V. and G.B.H. share senior authorship.

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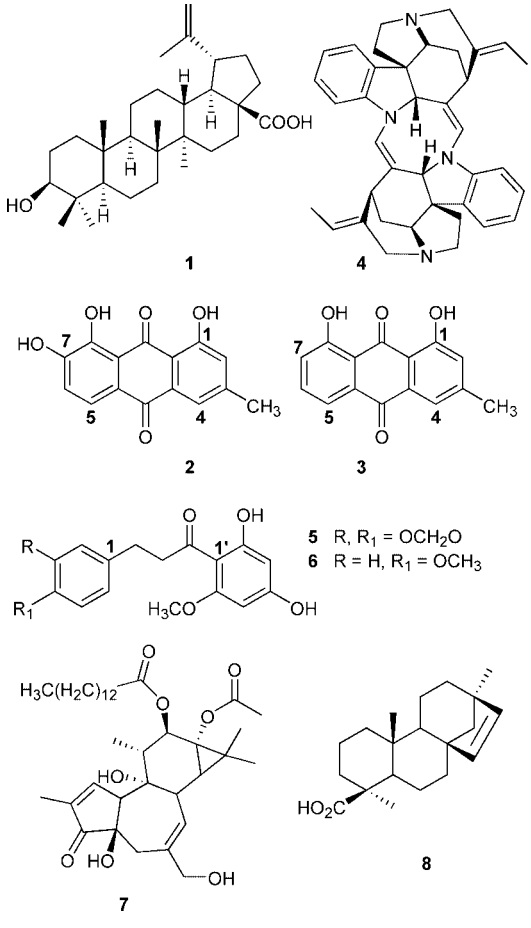
Table 1. Cytotoxicity of Compounds **1**–**8**[a]

	GI_{50} (μg/mL) in indicated cell line[b]									
compound	3T3	H460	ME180	DU145	MCF-7	M-14	HT-29	PC3	K562	Vero
1	>62.5	29.7	27.8	35.3	>62.5	32.6	30.9	31.3	10.8	30.6
2	10.3	4.8	13.2	7.9	7.3	13.6	6.5	8.7	12.5	NA
3	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5
4	1.5	1.6	1.9	4.5	2.0	1.6	<1.0	2.1	<1.0	2.7
5	27.8	27.4	NA	22.4	18.9	23.4	27.6	24.5	8.4	28.4
6	42.5	44.7	NA	41.7	22.9	40.3	35.5	46.3	7.4	40.4
7	0.01								0.00012	
8	>62.5								>62.5	

[a] The maximum concentration investigated was 62.5 μg/mL. [b] 3T3, BALB/3T3 clone A31 embryonic mouse fibroblast cells; H460, human large cell lung cancer; ME180, human cervical carcinoma; DU145, human prostate carcinoma; MCF-7, human breast adenocarcinoma; M-14, human melanoma; HT-29, human colon adenocarcinoma; PC3, human prostate adenocarcinoma; K562, human chronic myelogenous leukemia cells; Vero, normal African green monkey kidney epithelial cells.

emodin (**2**) as the main cytotoxic metabolite in *P. sellowii*. Its highest cytotoxic activity was found against the H460 cell line (GI_{50} = 4.8 μg/mL, Table 1). The anthraquinone chrysophanol (**3**) was also isolated, showing no significant cytotoxic activity with a GI_{50} > 62.5 μg/mL. Compounds **2** and **3** did not show anti-MTB activity at 25 μg/mL. The structures of these compounds were identified by comparison of their spectroscopic data with those reported previously.[10]



Strychnos comprises around 200 species, and several of these are used in traditional medicine for the treatment of ulcers, wounds, swellings, leprosy, cholera, and even rabies.[10] The Aguaruna community use *S. mitscherlichii* bark extract pasted on dart tips as a poison for hunting, while in Colombia and elsewhere in South America it is an ingredient of curare used in the same way to kill animals.[12] In Guyana, a bark infusion of the liana, known as "devildoer", is drunk as an aphrodisiac.[13] The bioassay-guided fractionation of the chloroform extract led to the isolation of the cytotoxic, dimeric tertiary indole alkaloid bisnordihydrotoxiferine (**4**), which was also found to be the main metabolite present in the extract. The highest cytotoxic activities of **4** were found against the HT-29 and K562 cell lines (GI_{50} < 1.0 μg/mL, Table 1). Compound **4** showed a moderate anti-MTB activity, with a MIC value of 12.5 μg/mL against the $H_{37}Rv$ strain and 6.25 μg/mL against the MDR-MTB strain. Its identification was completed by comparing its spectroscopic data with those values reported previously.[14]

Iryanthera juruensis is distributed widely in the tropical rainforests of South America. In the Aguaruna community, where this plant is known as "untush tsempu", the resin of this tree is applied to treat aphthous stomatitis. A previous study on *I. juruensis* identified antioxidant principles.[15] In the present work, the bioassay-guided fractionation of the diethyl ether extract of *I. juriensis* led to the isolation of two dihydrochalcones, 2′,4′-dihydroxy-6′-methoxy-3,4-methylenedioxydihydrochalcone (**5**) and 2′,4′-dihydroxy-4,6′-dimethoxydihydrochalcone (**6**), as the cytotoxic active metabolites. Their most potent cytotoxic activity was found against the K562 cell line (GI_{50} = 8.4 and 7.4 μg/mL, respectively). Compounds **5** and **6** did not show anti-MTB activity at 25 μg/mL. The structures of these compounds were identified by comparison of their spectroscopic data with those reported previously.[16]

Croton alnifolius can be found in South America from northeastern Ecuador through the Peruvian mountains and foothills to adjacent Bolivia. Some species of *Croton* are used commonly in traditional medicine in Africa, Asia, and South America to treat a wide variety of health problems, especially as antiulcer, antitumor, and antimicrobial agents.[17] The bioassay-guided fractionation of the ethanol extract of *C. alnifolius* against the K562 cell line led to the identification of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA, **7**) as its cytotoxic constituent (GI_{50} = 0.12 ng/mL). The diterpenoid monoginoic acid (**8**) was also isolated as the main secondary metabolite present in this plant, exhibiting no significant cytotoxic activity (GI_{50} > 62.5 μg/mL). TPA is an expensive and well-documented tumor-promoting and cytotoxic agent[18] that is currently obtained by isolation from *Croton tiglium* seed oil. The isolation of TPA from *C. alnifolius* represents a potentially alternative natural source for this important pharmacological tool. The structures of these compounds were identified by comparing their spectroscopic data with those reported previously.[19]

Table 1 shows the cytotoxicity of the compounds isolated from the different plants. Excluding phorbol ester **7**, the alkaloid **4** was found to be the most active, with GI_{50} values that are in some cases up to 30 times lower than the corresponding ones for the triterpene **1**. Even though we identified potent activity for compound **4**, it has limited selectivity or differential cytotoxicity. After comparing the structures of compounds **2** and **3**, the moderate and nonselective cytotoxicity of **2** could be due to the presence of the C-7 hydroxy group of this anthraquinone, since no cytotoxicity was found for **3** at 62.5 μg/mL.

In summary, the collection of the ethnomedicinal information of the Aguaruna community from the Peruvian jungle, together with

an extensive multidisciplinary collaborative effort from scientific groups from two different continents, has led to the collection of close to 4000 plant species and the screening of over 2500 plant extracts; this constitutes one of the world's largest targeted inventory of tropical medicinal flora. The present study focused on the identification of the main cytotoxic constituents of five species belonging to different—and in some cases poorly studied—plant families. This led to the isolation of bioactive principles with different ranges in cytotoxicity and in all cases starting from a small amount of crude extract.

Experimental Section

General Experimental Procedures. ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), and 2D NMR spectra were recorded on a Varian INOVA 500 MHz spectrometer, using TMS as internal standard. GC-EI/MS analyses were obtained on a Varian Saturn 2000 workstation. HRMS data were obtained at the Nebraska Center for Mass Spectroscopy. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was obtained in a Perspective Biosystems instrument, using 2,5-dihydroxybenzoic acid in methanol−water as matrix (1:1, 20 mg/mL; Aldrich). Preparative TLC was performed on Silicycle silica gel 60 F_{254} glass plates (1.0 mm layer thickness). Column chromatography was carried out over Silicycle silica gel (230–400 mesh) or over Amersham Biosciences Sephadex LH-20. Fractions obtained from column chromatography were monitored on Merck silica gel 60 F_{254} aluminum sheets. HPLC was performed on a Nova-Pak column 6 μm (7.8 × 300 mm, Waters) or SymmetryPrep C_{18} column, 7 μm (19 × 300 mm, Waters).

Plant Material. *Doliocarpus dentatus* was collected at Río Domingusa, province of Condorcanqui, Amazonas Department, Perú, in September 1997 (voucher accession number, Lewis et al. 18577). *Picramnia sellowii* was collected at Imaza and Kunchin, province of Bagua, Amazonas Department, Perú, in March 1997 (voucher accession number, Castro et al. 17552). *Strychnos mitscherlichii* was collected at Puerto Tunduza, province of Condorcanqui, Amazonas Department, Perú, in September 1997 (voucher accession number, Lewis et al. 18214). *Iryanthera juruensis* was collected at Yamayakat, Quebrada Kuzu, province of Condorcanqui, Amazonas Department, Perú, in November 1996 (voucher accession number, Albán et al. 09133). *Croton alnifolius* was collected between Olmos and Jaén at hwy. km 20–21, Cajamarca Department, Perú, in March 1997 (voucher accession number, Lewis et al. 17332). All vouchers specimens are kept at the Museo de Historia Natural, Universidad Nacional Mayor de San Marcos (UNMSM), Lima, Perú, and at the Missouri Botanical Garden (MBG), St. Louis, MO. All plant material was identified by taxonomic staff at the MBG.

Extraction and Isolation. Ground, dried lianas (50 g) of *D. dentatus* were extracted with 95% ethanol, affording 8.7 g of extract. An aliquot of the extract (4.6 g) was partitioned between diethyl ether and water. The diethyl ether-soluble extract, in which the cytotoxic activity was concentrated, was chromatographed on a silica gel column using a hexane−chloroform gradient. Six fractions (A−F) were obtained and evaluated for their cytotoxicity. An aliquot (100 mg) of the most active fraction (E) was chromatographed on a silica gel column using a hexane−chloroform gradient, resulting in the isolation of betulinic acid (**1**, 10 mg). Physical and spectroscopic data matched those in the literature.[7]

Powdered, dried leaves (50 g) of *P. sellowii* were extracted with 95% ethanol, affording 8.3 g of extract. An aliquot of the extract (4.7 g) was partitioned between hexane, diethyl ether, and water. The organic-soluble extract, which exhibited cytotoxic activity, was chromatographed on a silica gel column using a hexane−chloroform gradient. Four fractions (A−D) were obtained and evaluated for their cytotoxicity. Fraction C (245 mg), which concentrated the cytotoxic activity, was dissolved in chloroform and extracted three times with 5% aqueous HCl, with the aqueous layer then taken to pH 10 using 20% aqueous NaOH and extracted three times with chloroform. The organic layer was dried and redissolved in chloroform−MeOH and stored overnight below 0 °C, and the resultant orange needles of nataloe-emodin (**2**, 4.3 mg) were filtered and washed with MeOH. Fraction B (81 mg) was recrystallized from methanol, giving chrysophanol (**3**, 1.4 mg). Physical and spectroscopic data matched those in the literature.[10]

Ground, dried root bark and inner bark (65 g) of *Strychnos mitscherlichii* was extracted with 95% ethanol, affording 4.2 g of extract. An aliquot of the extract (975 mg) was partitioned between dichloromethane and water. The dichloromethane-soluble extract, which concentrated the biological activities, was subjected to a silica gel column chromatography using a chloroform−MeOH gradient. Five fractions (A−E) were obtained and evaluated for their cytotoxicity. The most active fraction (C, 55 mg) was purified by reversed-phase preparative TLC using MeOH−acetonitrile−H_2O−diethylamine (12:4:2:1; two developments), resulting in the isolation of bisnordihydrotoxiferine (**4**, 9.0 mg). Physical and spectroscopic data matched those in the literature.[14]

Ground, dried bark (69 g) of *I. juruensis* was extracted with 95% ethanol, affording 6.9 g of extract. An aliquot of the extract (4.1 g) was partitioned between diethyl ether and 40% MeOH in water. The diethyl ether-soluble extract, which concentrated the cytotoxic activity, was chromatographed on a silica gel column using a hexane−chloroform−MeOH gradient. Six fractions (A−F) were obtained and evaluated for their cytotoxicity. The most active fraction (E, 63 mg) was chromatographed on a silica gel column using a hexane−ethyl acetate gradient. Two fractions were obtained (E_1, E_2) and evaluated for their cytotoxicity. An aliquot of fraction E_2 (10.0 mg), which concentrated the cytotoxic activity, was purified by reversed-phase HPLC eluted with water−MeOH−chloroform (7:5:1, flow rate 9.0 mL/min), to afford 2′,4′-dihydroxy-6′-methoxy-3,4-methylenedioxydihydrochalcone (**5**, 4.3 mg, t_R = 62.5 min) and 2′,4′-dihydroxy-4,6′-dimethoxydihydrochalcone (**6**, 2.0 mg, t_R = 66.0 min). Physical and spectroscopic data matched those in the literature.[16]

Powdered, dried fruits (136 g) of *Croton alnifolius* were extracted with 95% ethanol, affording 2.6 g of extract. An aliquot of the extract (1.8 g) was dissolved as much as possible in chloroform, obtaining two fractions. The chloroform-soluble portion was subjected to Sephadex LH-20 column chromatography using a dichloromethane−methanol gradient. Three fractions (A−C) were obtained and evaluated for their cytotoxicity. Fraction B (1.0 g), which concentrated the cytotoxic activity, was chromatographed on a silica gel column using a chloroform−methanol gradient. Seven fractions (B_1−B_7) were obtained and evaluated for their cytotoxicity. The most active fraction (B_5, 90 mg) was subjected to Sephadex LH-20 column chromatography using a dichloromethane−methanol gradient. Four fractions (B_{5A}−B_{5D}) were obtained. Fraction B_{5C} (35 mg), which concentrated the cytotoxic activity, was purified twice by normal-phase preparative TLC using diethyl ether−hexane (20:1) and then by normal-phase HPLC eluted with chloroform−MeOH (98:2, flow rate 4.5 mL/min) to afford 12-*O*-tetradecanoylphorbol-13-acetate (**7**, 1.6 mg, t_R = 10.5 min). Successive recrystallizations of the chloroform-soluble portion, using *n*-hexane−diethyl ether and freezing, afforded monoginoic acid (**8**, 23.6 mg). Physical and spectroscopic data matched those in the literature.[19]

Cell Growth Inhibition Bioassay. The crude extract and fractions were screened for cytotoxicity against a panel of human tumor cell lines and two nontumorogenic cell lines, using previously reported methodologies[4] at a maximum concentration of 62.5 μg/mL. Briefly, growth inhibition was evaluated by preparing serial dilutions of each fraction or compound and incubating the cells in 96-well plates in the presence or absence of these fractions for 48 h at 37 °C. Appropriate solvent controls were tested for comparison. The percent inhibition of cell growth relative to the control was evaluated colorimetrically using a sulforhodamine B dye according to a published procedure[20] by comparison to the control. The GI_{50} value was defined as the concentration of test sample resulting in a 50% reduction of absorbance as compared with untreated controls that received a serial dilution of the solvent in which the test samples were dissolved, and was determined by linear regression analysis.

Anti-MTB Activity Bioassay. The five cytotoxic compounds were assayed using the tetrazolium microplate assay (TEMA) as described previously.[21]

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Supporting Information Available: Figure S1 and fractionation schemes. This material is available free of charge via the Internet at http://pubs.acs.org.

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