Aegicerin, the First Oleanane Triterpene with Wide-Ranging Antimycobacterial Activity, Isolated from *Clavija procera*

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Received December 29, 2005

An ethanol extract of the Peruvian plant *Clavija procera*, a member of the rare Theophrastaceae family, was fractionated using a colorimetric bioassay-guided protocol against *Mycobacterium tuberculosis* (MTB), yielding the oleanane triterpenoid aegicerin (**1**) as the active constituent. Its MIC values ranged between 1.6 and 3.12 μg/mL against 37 different sensitive and resistant MTB strains (1 H37Rv, 21 susceptible clinical isolates, 2 INH-resistant clinical isolates, and 13 MDR clinical isolates).

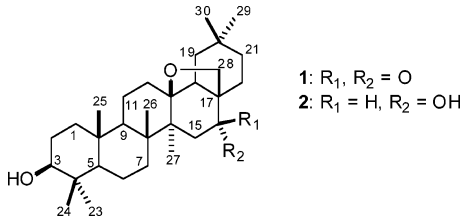
Tuberculosis (TB) is the cause of 2 million deaths per year worldwide, with 98% occurring in developing countries. One of the highest incidence rates in the Americas occurs in Peru (228 cases per 100 000 population in 1999).[1,2] The HIV epidemic has worsened this situation, especially in resource-poor countries. In the developing world, about 50% of HIV seropositive individuals may be co-infected with TB.[3] Inadequate, incomplete, or improperly supervised treatment regimens and co-infection with HIV have caused the emergence of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* (MTB).[4] Due to the urgent need to discover new drugs for the treatment of tuberculosis, several colorimetric methods for rapid screening of pure compounds or crude natural product extracts have been developed.[5−7] The microplate Alamar blue assay (MABA) is a rapid, nonradioactive, and inexpensive method that is based on the color change of the redox dye Alamar blue for the detection of viable *M. tuberculosis*.[8,9] Members of our group have developed a variant of this assay that uses tetrazolium bromide instead of the more expensive Alamar blue dye.[10] This tetrazolium microplate assay (TEMA) determines MIC values as quickly (6 to 7 days) and accurately as the MABA procedure.

Northern Peru, particularly the eastern slopes of the Andes Mountains and adjacent upper Amazon basin, the area from which *Clavija procera* B. Stahl (Theophrastaceae) originates, is exceedingly rich in diverse woody plants. This area has evolved into the richest source of biodiversity, exceeding the plant diversity of sub-Saharan Africa and Southeast Asia.[11] Only a small percentage of these Peruvian species have been investigated chemically and/or biologically. Using the TEMA assay, a group of 591 ethanol extracts from Peruvian plants were screened for antimycobacterial activity in vitro against the sensitive MTB H37Rv strain.[12] Thirty seven (6.3%) extracts showed MIC values ≤ 100 μg/mL. The most active corresponded to the extract from the stems and bark of *C. procera* with a MIC of 12.5 μg/mL. The genus and family of this species are poorly known. Indeed, no previous phytochemical or pharmacological investigations have been reported for *C. procera*. This plant is traditionally used by the Aguaruna people for the wound healing of cutaneous leishmaniasis. The bioassay-guided isolation of the ethanol extract of *C. procera* yielded an active fraction (MIC = 3.12 μg/mL), for which final purification resulted in the isolation of aegicerin (**1**) and protoprimulagenin A (**2**). Compounds **1** and **2** were previously isolated from *Aegiceras* spp.[13,14] and from *Embelia schimperi*.[15]

Table 1. Antimycobacterial Activity of INH, RIF, and Aegicerin (**1**)

	MIC (μg/mL)		
strains (number of strains tested)	INH[a]	RIF[b]	aegicerin (**1**)
Mycobacterium tuberculosis			
H37Rv	0.125	0.063	3.1
susceptible clinical isolates (21)	0.125	0.063	1.6−3.1
INH-resistant clinical isolates (2)	2−4	0.063−0.125	1.6−3.1
MDR clinical isolates (13)	4−>32	2−>16	1.6−3.1

[a] INH = isoniazid. [b] RIF = rifampin.



Aegicerin (**1**) was investigated for its in vitro cytotoxic activity against Vero cells (GI_{50} >40 μg/mL) using previously reported methodologies.[16−18] (The GI_{50} is the concentration at which aegicerin inhibits the growth of cells by 50%.) Evaluation of the antimycobacterial activity in vitro was performed using the TEMA method.[10] The skeleton of **1** and **2** is not very common; it consists of an oleanane-type pentacyclic triterpene skeleton with a methyleneoxy bridge between C-13 and C-17. Aegicerin (**1**) was tested against a total of 37 different sensitive and resistant MTB strains (1 H37Rv, 21 susceptible clinical isolates, 2 INH-resistant clinical isolates, and 13 MDR clinical isolates). Its MIC ranged between 1.6 and 3.12 μg/mL (Table 1). Conversely, protoprimulagenin A (**2**) was inactive (MIC = 200 μg/mL).

Comparison of structures **1** and **2** and their MIC values demonstrates that the presence of a carbonyl group at C-16 is crucial

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10.1021/np050554l CCC: $33.50
Published on Web 03/15/2006

for antimycobacterial activity. Although a number of triterpenes possessing mild to strong antimycobacterial activity are known,[19−29] this work marks the first time that an oleanane-type triterpene has shown such a consistently high level of activity against a large panel of both sensitive and resistant strains. While aegicerin (**1**) has been previously isolated, its wide-ranging anti-TB potential has not been realized.[30] In summary, using an inexpensive and reproducible colorimetric assay, a potent antimycobacterial triterpenoid, aegicerin (**1**), was isolated from a plant species belonging to a rare genus and family. Aegicerin (**1**) was found to possess excellent activity against a large number of MDR-MTB strains.

Experimental Section

General Experimental Procedures. The structures of the isolated compounds were identified by nuclear magnetic resonance (NMR; Varian Inova 500), ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), and 2D-NMR analysis in $CDCl_3$; by mass spectrometry (HREIMS at the Nebraska Center for Mass Spectrometry); and by comparison of spectroscopic data with those reported in the literature.[11] Column chromatography was carried out over silica gel (70−230 mesh, Merck). Fractions obtained from column chromatography were monitored by TLC (silica gel 60 F_{254}, Merck). Preparative TLC was carried out on Merck silica gel F_{254} plates (20 × 20 cm, 0.5 mm thick).

Plant Material. *Clavija procera* (Theophrastaceae) was collected near the community of Sukutim, province of Bagua, Amazonas Region, in August 1998 (vouchers #19191 kept at the Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Lima, Peru, and Missouri Botanical Garden, St. Louis, MO). The plant was identified by one of us (W.H.L.)

Extraction and Isolation. Dried stems and bark of *C. procera* (110 g) were extracted with 95% ethanol. This ethanol extract (6.25 g) was partitioned with the aid of solvents of different polarities. The activity was concentrated in the solvent system corresponding to 90% methanol−water. After concentration, this purified extract was chromatographed on a silica gel column using a hexane−ethyl acetate gradient. Five fractions (A−E) were obtained and evaluated for anti-MTB activity in vitro. The most active fraction (C, MIC = 3.12 μg/mL) was purified by silica gel preparative TLC using hexane−chloroform−methanol (25:10:2; triple development), resulting in the isolation of aegicerin (**1**, 5.7 mg, 0.005% yield) and protoprimulagenin A (**2**, 2.1 mg, 0.002% yield).

Bioassays. For the preparation of the inoculum, a suspension of MTB was made by mixing growth from slants (20−30 days old) with 100 μL of Tween 80 into 0.2% bovine serum albumin (Sigma Chemical Co., St. Louis, MO). Turbidity of the suspension was then adjusted to a McFarland standard No. 1 (3×10^7 CFU/mL) by adding Tween 80 and bovine serum albumin. The bacterial suspension (300 μL) was further transferred to 7.2 mL of 7H9GC broth (4.7 g of Middlebrook 7H9 broth base (Difco, Detroit, MI), 20 mL of 10% glycerol, 1 g of Bacto Casitone (Difco), 880 mL of distilled water, 100 mL of OADC (oleic acid, albumin, dextrose, catalase) (Remel, Lenexa, KS)). For the bioassay, the extracts or pure compounds were resuspended in DMSO at a concentration of 4 mg/mL (stock solution). These stock solutions were further diluted with appropriate volumes of 7H9GC broth to yield final concentrations of 0.4 to 25 μg/mL. Final drug concentration ranges of standard antibiotics used as positive controls were 0.125 to 32 μg/mL for isoniazid and 0.063 to 16 μg/mL for rifampin. The plant extract or drug (100 μL) was mixed in the wells with 100 μL of bacterial inoculum, resulting in a final bacterial concentration of approximately 1.2×10^6 CFU/mL. The wells in column 11 served as inoculum-only controls. Solvent (DMSO) was included in every experiment as a negative control. The plates were sealed in plastic bags and then incubated at 37 °C for 5 days. On day 5, 50 μL of the tetrazolium−Tween 80 mixture {1.5 mL of tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Aldrich Chemical Co., Milwaukee, WI) at a dilution of 1 mg/mL in absolute ethanol and 1.5 mL of 10% Tween 80} was added to the wells, and the plate was incubated at 37 °C for 24 h. After this incubation period, the growth of the microorganism was visualized by the change in color of the dye from yellow to purple. The tests were carried out in triplicate. MIC is defined as the lowest drug or plant extract concentration that prevents the aforementioned change in color.

Acknowledgment. The authors are grateful to the Iberoamerican Program for Science and Technology (CYTED), Project X.11:PIBATUB (grant to R.R.), to the NIH and NSF (ICBG grant U01TW00331 to W.H.L., G.L., and A.J.V.), and the University of Louisville, Office of the Vice-Provost for Research (G.B.H.), for their financial support. We acknowledge the spectroscopic support provided by Dr. N. Stolowich, Department of Chemistry, University of Louisville. Our gratitude is extended to the Aguaruna people for generously sharing their traditional medicinal information. In particular, we acknowledge the assistance of organizational/clan leaders, Apus, and members of OCCAAM, FAD, FECONARIN, and OAAM.

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NP050554L