Antibacterial Activity of Lonchocarpol A

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Lonchocarpol A, a flavanone, demonstrates *in vitro* inhibitory activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*. This activity is antagonized by mouse plasma, which may account for its lack of *in vivo* activity. This compound demonstrates no differentiation with respect to the inhibition of RNA, DNA, cell wall, and protein synthesis.

During the course of screening natural products for antimicrobial activity, an extract, prepared from the last larval stage of *Melipotis perpendicularis* (Noctuidae), was designated a candidate for further isolation work based on its activity in a standard agar diffusion assay.[1] The chemistry of *M. perpendicularis* has not been described previously. The sample was provided by our collaborators at the Instituto Nacional de Biodiversidad (INBio) as part of our ongoing contractual agreement in bioprospecting/drug discovery. After this finding, an examination of the INBio database revealed 11 additional *M. perpendicularis* samples as well as 24 other *Melipotis* species in our extract library. These samples were composed of various life-cycle stages of prepupa, pupa, larvae, penultimate larvae, and adults, but none of these samples was found to exhibit antimicrobial activity. At this point the connection between larvae and host plant was made. Extracts prepared from larvae feeding on other plant hosts were inactive. It was, therefore, established that the activity was the direct result of the larvae's feeding on the leaves of the host plant *Lonchocarpus minimiflorus* (Leguminosae) and not a consequence of insect metabolism. The active component lonchocarpol A (senegalensien) [2,3] was readily purified from crude extracts of both insect and plant by first employing SPE (SiO_2) followed by preparatory C18 HPLC. The compound was fully characterized by HRMS, 1D and 2D NMR data analyses. A literature search revealed a match with the previously described lonchocarpol A.[2,3]

Lonchocarpol A, a flavanone, was originally isolated from bark samples of *Lonchocarpus* and *Erythrina* species[2,3] and more recently from bark and root samples of *Citrus*, *Lupinus*, and *Sophora* species.[4−8] This compound has been reported to have insecticidal and anticancer activity[9] but no antifungal activity.[7] However, there has been no antibacterial data reported to date. In this communication, we describe the isolation and antibacterial activity of this compound.

Table 1 presents the MIC values of lonchocarpol A for *Staphylococcus aureus*, *Enterococcus faecium*, and *Mycobacterium smegmatis*. Also indicated is the MIC value for *Bacillus megaterium*, which was determined for the purpose of labeling for macromolecular synthesis.

Table 1. Minimum Inhibitory Concentration (MIC) of Lonchocarpol A

test organism	MIC (μg/mL)	
	minus plasma	plus plasma[a]
Staphylococcus aureus[b]	0.78−1.56	≥100
Enterococcus faecium[c]	0.78−1.56	≥100
Mycobacterium smegmatis	>100	>100
Bacillus megaterium	1.0−2.0	

[a] 45% final concentration. [b] Methicillin resistant. [c] Vancomycin resistant.

The initial MIC values for *S. aureus* and *E. faecium* were 0.78 to 1.56 μg/mL, which highlighted this compound as a possible therapeutic agent. Subsequent MIC determinations, however, indicated antagonism by serum, which significantly elevated MIC values to ≥100 μg/mL. The MIC value for *M. smegmatis* was >100 μg/mL and deemed not significant. The MIC value for *B. megaterium* was 1.0−2.0 μg/mL. The serum effect on the MIC value for *M. smegmatis* could not be determined at the concentrations tested and was not investigated for *B. megaterium*.

A target organ assay was employed to determine the effective dose of lonchocarpol A. Lonchocarpol A exhibited potent antistaphylococcal activity *in vitro* but, unfortunately, was not efficacious *in vivo*. Clearance of *S. aureus* (Smith strain) from the kidneys of infected mice treated with lonchocarpol A was not significantly reduced from that of sham-treated control mice. This may be due in part to its high affinity to bind to serum (as exhibited by higher MIC values in the presence of serum). Also, this compound appeared to be somewhat toxic at doses of 50 and 10 mg/kg/day.

Further biological characterization of this compound, which included the reduction of 3-4,5 dimethylthiazol-2,5 diphenyl tetrazoleum (MTT) and the exclusion of trypan blue by mouse L-1210 cells, were used to assess the cytotoxicity of lonchocarpol A. The IC_{50} for MTT reduction and trypan blue exclusion are >10 and 10 μg/mL, respectively. In addition, red blood cell hemolysis was also measured. The minimum lytic concentration (MLC) of this compound was determined to be ≥100 μg/

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mL. These data indicate that this compound is neither cytotoxic nor red blood cell hemolytic at MIC concentrations.

The effect of lonchocarpol A on macromolecular synthesis was also investigated. Whole-cell labeling was used to determine whether the synthesis of a specific macromolecule was preferentially reduced after the exposure of *B. megaterium* to an MIC level of lonchocarpol A. The results demonstrate that lonchocarpol A inhibits all types of macromolecular synthesis at similar concentrations. The IC_{50} values for RNA, DNA, cell wall, and protein synthesis are 1.9, 3.9, 1.9, and 2.5 μg/mL, respectively, a range similar to the MIC.

The biological activity of lonchocarpol A indicates that plants should continue to be exploited as a source of biologically active compounds. In addition, compounds that have been previously reported in the literature should also be evaluated as possible therapeutic agents for emerging bacterial infections.

Experimental Section

General Experimental Procedures. HRMS was obtained with a VG AutoSpec-Q mass spectrometer using a resolving power of 5000 (10% valley definition). The analysis was performed in LSIMS mode using glycerol as the matrix. Analytical HPLC analyses were carried out on a Hewlett−Packard 1090 system (UV 210 nm), while a Waters DeltaPrep 3000 system was used for the semipreparative isolation work.

NMR spectra were recorded on a Varian VXR-500S spectrometer. The sample was dissolved in CD_2Cl_2 (99.96 atom-% D; Merck Isotopes) containing a small amount of TMS as an internal reference. Standard pulse sequences were used for COSY, NOESY, HMQC, and HMBC experiments.

Isolation of Lonchocarpol A from Moth Larvae. Approximately 20 g (fresh wt) of insect material was collected in Costa Rica in May 1993. The voucher sample is deposited at the Instituto Nacionale de Biodiversidad, sample 93-LFCHD-084. The larvae, stored at −20 °C prior to lyophilization, weighed 3.4 g after drying. The mass was then placed into a small glass column with frit and extracted with a series of three solvents—hexane, CH_2Cl_2−MeOH (1:1), and H_2O. After solvent removal *in vacuo*, the final residue weights were 0.47, 0.38, and 0.62 g, respectively. Only the CH_2Cl_2−MeOH extract was active. A 161-mg portion of this extract was subjected to Si gel (6.4 g, 70−230 mesh) solid-phase extraction (SPE) eluting with hexane, CH_2Cl_2, EtOAc, and MeOH to yield 1.8 mg, 15.4 mg, 10.2 mg, and 105.9 mg, respectively. Both the CH_2Cl_2 and EtOAc fractions were active and were subsequently combined and subjected to HPLC (1 × 3 mg, 3 × 5 mg) using a Vydac C_{18}, 10 μ, 22.5 mm × 250 mm column employing a linear MeCN−H_2O (0.1% TFA) 12 mL/min gradient starting 20% MeCN and ending 80% MeCN over 60 min. Lonchocarpol A (3.6 mg, t_R 39 min) appeared as a greenish yellow oil after solvent removal.

Isolation of Lonchocarpol A from Plant Source. Leaves of *L. minimiflorus* were collected in Costa Rica in February 1995. A voucher specimen, 102-41.0L, is maintained at INBio. Approximately 101 g of the dried *L. minimiflorus* leaves were placed into a 1-L glass percolator and extracted with 95% EtOH. The resultant 7.33 g residue was partitioned between hexane and 10% aqueous MeOH, with the resultant MeOH residue partitioned again between CH_2Cl_2 and H_2O. The residues weighed 1.97, 1.33, and 3.56 g, respectively. The isolation procedure used above was duplicated for the leaf extract of *L. minimiflorus*, except that the compound resided entirely in the EtOAc fraction from SPE. Five runs of 5 mg each of the EtOAc residue were performed via C_{18} HPLC to obtain 8.8 mg lonchocarpol A.

Lonchocarpol A: greenish yellow oil; HRFABMS *m/z* $[MH]^+$ 409.2090 ($C_{25}H_{29}O_5$ requires 409.2015).

Minimal Inhibitory Concentration (MIC) Determination. All procedures were performed under sterile conditions. Minimal inhibitory concentrations (MICs) were determined in triplicate in a broth microtiter susceptibility assay [Brain Heart Infusion broth (BHI); Difco]. Lonchocarpol A was dissolved in DMSO and diluted to 1.0 mg/mL in 10% DMSO. From this initial concentration, 10 additional serial two-fold dilutions were made in the same diluent in a microtiter plate, resulting in a final volume of 20 μL in each well. Stationary-phase cultures were diluted in a two-fold concentration of BHI to approximately 10^5 cfu/mL; 90 μL were added to each well along with either 90 μL of sterile mouse plasma (Pel-freez Biologicals) or distilled H_2O. The plates were incubated at 37 °C for 24 h and visually inspected for growth. The MIC was defined as the lowest concentration of compound preventing any visible growth.[10]

The MIC determination for *B. megaterium* Merck bacterium (Mb) 410 was also determined in a broth microtiter susceptibility assay. Lonchocarpol A was diluted in sterile distilled H_2O to 512 μg/mL. From this initial concentration, 10 additional serial two-fold dilutions were made in sterile distilled H_2O in a microtiter plate, resulting in a final volume of 50 μL in each well. *B. megaterium* Mb 410 was grown to stationary-phase culture in LB medium[11] and diluted 10^{-3} in two-fold concentrated LB medium. The microtiter dish was inoculated with 50 μL of bacterial culture and incubated at 37 °C for 24 h and visually inspected for growth. The MIC was defined as above.

Cytotoxicity Testing. Cytotoxicity determinations using MTT were performed using mouse L-1210 cells grown in Fisher's Medium (Difco) plus 10% heat-inactivated fetal bovine serum. The assays were performed in microtiter plates as described by Carmichael *et al.*[12] and Twentyman and Luscombe.[13] Cell viability was also assessed using trypan blue exclusion; an equal aliquot of treated L-1210 cells and 0.4% trypan blue (Sigma) were mixed, and the total number of cells, as well as the proportion of viable (i.e., dye-excluding) cells, were determined by microscopic observation. In both MTT and trypan blue assays, the IC_{50} indicates the concentration of compound resulting in a viable cell number that is half the level of the control. The trypan blue assay was also used to determine the extent of lysis of L-1210 cells after a 24 h incubation.

Minimum Lytic Concentration (MLC). MLCs were determined in a liquid microtiter assay. A 1-mg/mL stock solution of lonchocarpol A was prepared in 25% DMSO−H_2O and kept at −80 °C. The thawed stock solution was diluted (1:8) to 125 μg/mL with a 5%

(v/v) dextrose solution. From this initial concentration, 11 additional serial two-fold dilutions were made in 4.38% dextrose solution in a microtiter plate, resulting in a final volume of 150 μL per well (125 to 0.06 μg/mL). Of a 4% solution (v/v) of defibrinated sheep blood (BBL) in 5% dextrose (v/v) 38 μL was added to each concentration of compound and mixed gently. The multiwell plate was covered and incubated at 25 °C and 175 rpm for 2 h. Hemolysis was demonstrated as full or partial clearing. The MLC was defined as the minimum concentration required to cause hemolysis.

Whole-Cell Labeling. The effect of lonchocarpol A on macromolecular synthesis was evaluated using whole-cell labeling methods in which *B. megaterium* Merck bacterium (Mb) 410 was grown in LB medium. The radioactive precursors used were [5,6-^3H]uracil (New England Nuclear) at 38.6 Ci/mmol for RNA inhibition, [6-^3H]thymidine (New England Nuclear) at 15 Ci/mmol for DNA inhibition, meso-[2,6-^3H]diaminopimelic acid (American Radiolabeled Chemicals) at 50 Ci/mmol for cell wall inhibition, and L-[4,5-^3H]leucine (Amersham) at 151 Ci/mmol for protein inhibition. The effect of lonchocarpol A on macromolecular synthesis was determined at the MIC level. *B. megaterium* Mb 410 was incubated to early log phase at 37 °C in LB containing 50 μg/mL uridine and 100 μg/mL L-lysine. The bacteria were concentrated by centrifugation and resuspended in two-fold concentrated LB at an optical density (660 nm) of 4. The culture (25 μL) was distributed into the wells of microtiter plates each containing 50 μL of test sample prepared in distilled H_2O and 25 μL of radioactive precusor prepared in two-fold concentrated LB medium containing 400 μg/mL of L-lysine. The medium containing [6-^3H]thymidine also contained 200 μg/mL uridine. The plates were incubated on a rotary shaker (New Brunswick Scientific) at 200 rpm and 37 °C. The incorporation of the precursors was terminated after 15 min by the addition of 25 μL of 25% trichloroacetic acid. The precipitated bacteria were collected on glass microfiber filtermats and washed with distilled H_2O with a cell harvester (Skatron). Radioactivity adsorbed to the filtermats was measured with a scintillation counter (Wallac Betaplate).

ED_{50} Determination. A target organ (kidney) assay utilizing *S. aureus* Mb 2865 (Smith strain) was employed to determine the effective dose of lonchocarpol A in mice. A frozen 1-mL aliquot of the organism was thawed and used to seed 49 mL of Brain Heart Broth (BHB, Difco). This culture was incubated at 35 °C for 10 h with shaking (250 rpm). The turbidity of this 10 h culture was adjusted to the turbidity of a 0.5 McFarland standard using the A-JUST meter (Abbott, approximately 1×10^7 cfu/mL). This adjusted culture was then further diluted 1:500 in fresh BHB yielding approximately 2×10^4 cfu/mL. When 0.5 mL was administered intraperitoneally to 20-g mice, the final infectious dose was equal to 1×10^4 cfu/mouse.

Lonchocarpol A was solubilized in 10% DMSO, further diluted in sterile distilled H_2O and administered subcutaneously three times-per-day for 1 day. When 0.5 mL of each dilution was administered subcutaneously, the final therapeutic doses were 50, 10, 5, and 0.5 mg/kg/day.

Mice were infected intraperitoneally with the inoculum described above. Therapy was initiated 5−10 min after infection with the total amount delivered in three subcutaneous doses on Day 0. Mice were observed for general health and mortality for 48 h after challenge. On Day 2, three mice per group were euthanized and both kidneys aseptically removed, placed in sterile Whirl-Pak bags (Nasco), weighed, and homogenized in 5 mL of sterile saline. Tissue homogenates were then serially diluted 100-fold in sterile saline and plated on mannitol salt agar (Difco). Plates were incubated for 48 h at 35 °C. Colony forming units per gram of tissue were determined and compared to counts from sham-treated control mice.

All animal procedures were performed in accordance with the highest standards for the humane handling, care, and treatment of research animals and were approved by the Merck Institutional Animal Care and Use Committee. The care and use of research animals at Merck meets or exceeds all applicable local, state, and federal laws and regulations.

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