# Antifungal Activity of a New Triterpenoid Glycoside from *Pithecellobium racemosum* (*M.*)

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***Purpose.*** In a continuation of our search for novel antifungal compounds from higher plants, the standard extract of the bark of *Pithecellobium racemosum* was found to have good activity against important AIDS-related opportunistic yeasts.

***Methods.*** The extract was subjected to bioguided fractionation using silica gel column chromatography which led to purification of triterpene glycosides. The structures of these compounds were determined by a combination of spectroscopic (IR, NMR, HRMS) and chemical methods.

***Results.*** Compound 1 is a new glycoside, 3-O[α-L-arabinopyranosyl (1-2)][α-L arabinopyranosyl (1-6)]2-acetoamido-2-deoxy-β-D-glucopyranosyl oleanolic acid and Compound 2 was identified as the known compound 3-O-[α-L-arabinopyranosyl (1-2)]α-L-arabinopyranosyl (1-6)] 2-acetamido-2-deoxy-β-D-glucopyranosyl echinocystic acid.

***Conclusions.*** Compound 1 is a new glycoside, 3-O-[α-L-arabinopyranosyl (1-2)]α-L-arabinopyranosyl (1-6)]-2-acetoamido-2-deoxy-β-D-glucopyranosyl oleanolic acid and exhibits moderate antifungal activity against *T. mentogrophytes*, *C. albicans* and *S. cerevisiae* with MIC values of 6.25, 12.5 and 12.5 μg/ml respectively.

**KEY WORDS:** *Pithecellobium racemosum*; antifungal; triterpene glycoside; oleanolic acid; Carbon-13 NMR.

## INTRODUCTION

The genus *Pithecellobium* (Leguminosae) consists of about 200 species. Only a few species have been investigated chemically, yielding saponins, alkaloids and flavonoids (1,2). The Brasilian species, *P. racemosum* is used extensively for furniture manufacturing due to the decay resistance of the wood. In our continuing search for novel antifungal compounds from natural sources, the bark of *P. racemosum* was collected near Manaus, Brasil and extracted with ethanol. The ethanol extract possessed significant antifungal activity and was fractionated to characterize the substance responsible for the observed antifungal activity.

## RESULTS AND DISCUSSION

Chromatography of the ethanolic extract of the stem bark of *P. racemosum*, followed by preparative thin layer chromatography afforded two compounds (1 and 2). The $^{13}$C-NMR spectrum (Table I) of each compound showed 48 carbon signals, suggesting that the compounds were saponin glycosides containing three sugars.

In both compounds, the IR spectral absorptions at $\upsilon_{max}$ 1650 and 1550 cm$^{-1}$, coupled with three characteristic signals in the $^{13}$C-NMR spectrum (23, 57 and 170 ppm) and a one proton broad doublet signal at 8.9 ppm suggested a $NHCOCH_3$. Comparison of the $^{13}$C-NMR signals of compound 1 with those of 3-O-(2-acetoamido-2-deoxy-β-D-glucopyranosyl)-oleanolic acid, which has been reported from *Pithecellobium cubense* (3) suggested that this unit represented a portion of the structure of 1. The fast atom bombardment mass spectrum (FAB MS) obtained in the negative ion mode showed a *quasi* molecular ion peak at m/z 922 [M-H]$^-$ and fragments at m/z 789, 657 and 455, which correspond to the successive elimination of two pentoses and one substituted sugar. Hydrolysis of compound 1 with 2N sulfuric acid yielded oleanolic acid (comparison with authentic sample, $^{13}$C-NMR, TLC). TLC analysis of the aqueous hydrolysate showed the presence of one product consistent with an aminosugar (pink color with ninhydrin), which had an Rf identical to authentic arabinose.

The sequence of sugar linkages in compound 1 (and 2, as shown in Figure 1) was deduced by several methods. Comparison of $^{13}$C-NMR spectral data of several model compounds with those of 1 provided support for the sequence shown in Figure 1 (4–9). In recent years an alternative approach, which does not involve chemical degradation, but rather relies on the measurement of carbon-13 spin-lattice relaxation time $T_1$ (10–12), has been used for the determination of the sequence of oligosaccharides. The $T_1$ values for the carbon atoms of 1 obtained in 0.19 M pyridine (d-5) solution at room temperature are shown in Figure 2. The average $NT_1$ values for the three sugars reflect their sequence [$NT_1$ terminal arabinose > $NT_1$ central arabinose > $NT_1$ inner sugar (N-acetylglucosamine)]. This sequence was further confirmed chemically by methylation analysis. The sample was methylated according to the Hakomori procedure (13), hydrolysed with acid, and the released, partially methylated, monosaccharides were analysed as their alditol acetates by GC/MS (14).

Compound 1 is a new glycoside, 3-O-[α-L-arabinopyranosyl (1-2)][α-L-arabinopyranosyl (1-6)]-2-acetoamido-2-deoxy-β-D-glucopyranosyl oleanolic acid. It exhibits moderate antifungal activity against *Trichophyton mentagrophytes*, *Candida albicans* and *Sacchaomyces cerevisiae*, with MIC values of 6.25, 12.5 and 12.5 μg/ml respectively. Amphotericin B was included as a positive control in each assay and exhibits a MIC vs. *T. mentagrophytes*, *C. albicans* and *S. cerevisiae* of 3.12, 1.56, and 0.39 μg/ml respectively.

Compound 2 was identified as the known compound 3-O-[α-L-arabinopyranosyl (1-2)][α-L-arabinopyranosyl (1-6)] 2-acetamido-2-deoxy-β-D-glucopyranosyl echinocystic acid, which has been isolated from *Albizzia anthelmintica* (15), based on comparison of the $^1$H and $^{13}$C-NMR spectral data with that reported.

## MATERIALS AND METHODS

### General

Melting points were determined in a Fisher-Johns digital melting point analyzer model 355 and were not corrected. Infra-

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**Table 1.** $^{13}C$ NMR Chemical Shifts in $C_5D_5N$

| Carbon | δ | Carbon | δ |
|---|---|---|---|
| 1 | 38.5 | GlcNAc | |
| 2 | 26.5 | 1 | 104.9 |
| 3 | 89.1 | 2 | 57.4 |
| 4 | 39.4 | 3 | 76.1 |
| 5 | 55.7 | 4 | 72.9 |
| 6 | 18.5 | 5 | 75.6 |
| 7 | 33.2 | 6 | 69.5 |
| 8 | 39.5 | Ara | |
| 9 | 47.9 | 1 | 102.5 |
| 10 | 36.9 | 2 | 79.7 |
| 11 | 23.5 | 3 | 72.6 |
| 12 | 122.5 | 4 | 67.8 |
| 13 | 144.7 | 5 | 64.5 |
| 14 | 42.1 | Ara | |
| 15 | 28.3 | 1 | 105.6 |
| 16 | 23.7 | 2 | 72.0 |
| 17 | 46.4 | 3 | 74.2 |
| 18 | 42.1 | 4 | 68.7 |
| 19 | 46.4 | 5 | 66.5 |
| 20 | 30.9 | | |
| 21 | 34.2 | | |
| 22 | 33.2 | MeCO-NH- | 23.0 |
| 23 | 28.1 | MeCO-NH- | 170.0 |
| 24 | 17.2 | | |
| 25 | 15.4 | | |
| 26 | 16.0 | | |
| 27 | 26.0 | | |
| 28 | 183.0 | | |
| 29 | 33.2 | | |
| 30 | 23.7 | | |

red spectra (IR) were taken as a KBr pellet on a Perkin Elmer 281B spectrometer. Ultraviolet spectra (uv) were recorded on a Perkin-Elmer Lambda 3B spectrometer using methanol as the solvent. NMR spectra were recorded on a Varian VXR (300 MHz). For both $^1H$ and $^{13}C$ nmr spectra, chemical shifts were expressed as ppm relative to TMS as internal standard (δ units). Fast atom bombardment mass spectra (FAB-MS) were obtained with a MAT 731 mass spectrometer. Alditol acetate derivatives of sugars were analysed as their acetates by gas chromatography on a Hewlett Packard Model 5890 with mass selective detector (MSD) on a DB-1 column, with a carrier of ultra pure helium. The injection port and detector temperatures were 250°C and the column temperature was programmed from 150–280°C at 5°C/min. Silica gel column chromatography was accomplished using MN silica gel 60 (70–270 mesh). Thin layer chromatography was accomplished using MN silica gel G/UV254 and detection of compounds was achieved by spraying with a prepared solution of EtOH/*p*-anisaldehyde/sulfuric acid/acetic acid (85:0.5:5:10).

### *Plant Collection*

The bark was collected near Manaus in the state of Amazonas, Brazil in June 1987. A voucher specimen has been deposited in the Herbarium of INPA (National Research Institute of Amazon), Manaus, Amazonas, Brazil.

### *Plant Extraction*

The dried, ground stem bark of *Pithecellobium racemosum* (1 Kg dry weight) was extracted by percolation with 95%

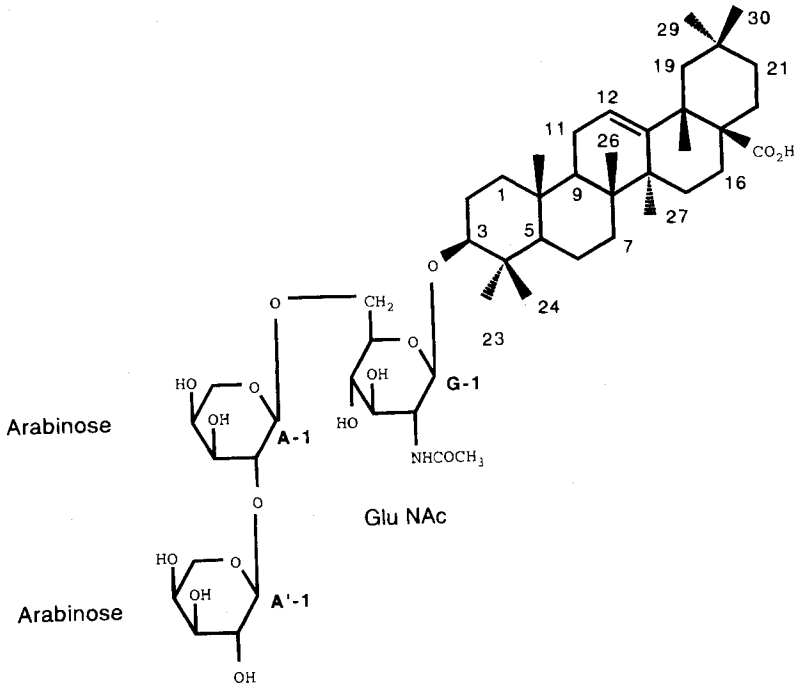


**Fig. 1.** Structure of 3-O-[α-L-arabinopyranosyl (1-2)][α-L-arabinopyranosyl (1-6)]-2-acetoamido-2-deoxy-β-D-glucopyranosyl oleanolic acid (Compound **1**).

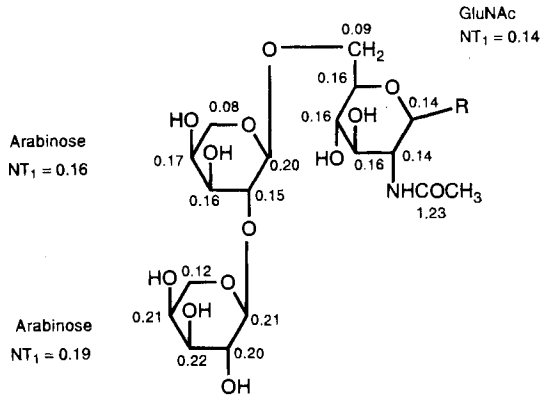


**Fig. 2.** Carbon-13 spin-lattice relaxation time ($T_1$) for glycone portion of compound **1**.

ethanol. The ethanol solution, upon evaporation under vacuum, yielded 19 g of a brownish resinous extract.

*Isolation of Saponins*

The crude extract (10 g) was adsorbed on silica gel (70–270 mesh, 200g) and chromatographed over silica gel (5 × 66 cm column) eluting with ethyl acetate (100%), followed by methanol/ethyl acetate (1:1) and, finally, methanol (100%). Fractions were collected on a volume basis (15 ml), and pooled based on TLC analyses to give two major fractions. Compound 1 was isolated from fractions 150–180 and Compound 2 was isolated from fractions 184–200. Further purification by small column chromatography and preparative TLC [$CHCl_3$-MeOH-$H_2O$ (7:13:8)] yielded pure 1 (300 mg) and 2 (100 mg). 3-O-(α-L-arabinopyranosyl (1-2))(α-arabinopyranosyl (1-6))-2-acetoamido-2-deoxy-β-D-glucopyranosyl oleanolic acid was obtained as an amorphous powder, mp 209 (dec); UV λmax, 210 nm; IR $\nu_{max}$ $cm^{-1}$: 1690 (COOH), 1650, 1550 (NHCO); FAB MS m/z ($C_{48}H_{77}NO_{16}$): 922 [M-H]-, 789 [M-132]$^-$, 657 [M-2 × 132]$^-$, 455 [M-(2 × 132)-222]$^-$; $^1$H NMR (300 MHz, $C_5D_5N$, TMS): δ 8.9 (br d, NH), 5.53 (1H, m, H-12), 2.18 (3H, s, MeCONH), 1.33, 1.31, 1.28, 1.22, 1.19, 0.94, 0.81 (21H, s, 7 × $CH_3$); $^{13}$C NMR (75 MHz, $C_5D_5N$, TMS): see Table I.

Compound 1 (40 mg) was hydrolysed with 2N sulfuric acid (10 ml) at 90°C temperature for 3 hrs. The hydrolysate was partitioned between $CHCl_3$ and 80% aqueous methanol. The $CHCl_3$ phase was dried under vacuum and purified by preparative TLC using ethyl acetate/hexane (1:4). The isolated sapogenin was indistinguishable from authentic oleanolic acid (Aldrich Chem. Co.) by TLC and $^{13}$C NMR spectroscopy. The aqueous phase was neutralized with $BaCO_3$, filtered and analyzed for sugars, using TLC analyses for comparison with authentic sugars.

Compound 1 (20 mg) was treated with NaH (100 mg) and MeI (1 mL) in dry DMSO (5 mL) and the mixture was kept overnight at room temperature. The reaction mixture was diluted with $H_2O$, extracted with $CHCl_3$ and the $CHCl_3$ layer was washed with water several times and evaporated to dryness.

*Reduction and Acetylation*

The product of permethylation was treated with 5 mL of 2N trifluoroacetic acid and heated for 5 hrs at 90°C. After complete hydrolysis, the reaction mixture was washed several times with water until neutralized, then dried under vacuum. The residue was dissolved in 100 μl of 1 M $NH_4OH$ and treated with 0.5 mL DMSO containing 15 mg of $NaBD_4$, and the mixture was incubated at 40°C for 2 hrs. The excess borodeuteride was destroyed with 150 mL of glacial acetic acid. The reduced sugars were acetylated by adding 100 μl of anhydrous 1-methylimidazole followed by 0.5 mL of acetic anhydride. Acetylation was complete in 20 min. at ambient temperature. Water (1.5 mL) was added to destroy unreacted acetic anhydride, and the reaction mixture was cooled to room temperature. Dichloromethane (1.5 mL) was added, and the permethylated alditol acetates were partitioned into the organic phase. The dichloromethane phase was washed with additional water to remove traces of DMSO and evaporated in a stream of nitrogen at ambient temperature. The derivatives were dissolved in 200 μl of dichloromethane and injected into a GC column for GC/MS analysis of the alditol acetate derivatives of the sugars.

## Biological Screening Procedures

Qualitative and quantitative antifungal evaluations were performed using the general antimicrobial bioassays described by Clark, *et al.* (16). The qualitative antifungal screening procedure consisted of testing the extracts, fractions and pure compounds against the following microorganisms, obtained from the American Type Culture Collection (ATCC): *Saccharomyces cerevisiae* (ATCC 9763), *Cryptococcus neoformans* (ATCC 32264), *Aspergillus flavus* (ATCC 9170), *Aspergillus fumigatus* (ATCC 26934), *Trichophyton mentagrophytes* (ATCC 9972) and *Candida albicans* (NIH B 311).

The antifungal activity was recorded as the width (in millimeters) of the inhibition zone (average radius) measured from the edge of the agar well to the edge of the inhibition zone after 24 h and 48 h of incubation. A standard antifungal agent, amphotericin B, was included in each assay as a positive control.

The minimum inhibitory concentration, using the two-fold serial macrobroth dilution assay, was determined for each compound that showed significant activity in the agar well diffusion assay. The MIC is recorded as the lowest concentration that inhibits visible growth after 48 h of incubation.

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