A Novel Antiprotozoal Aminosteroid from *Saracha punctata*

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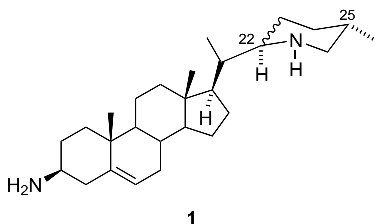
A new aminosteroid, 3β-amino-22,26-epiminocholest-5-ene named sarachine (**1**), and two known flavonoids, eriodictyol (**2**) and 7-*O*-β-D-glucopyranosyl-eriodictyol (**3**), were isolated from the leaves of *Saracha punctata*. The alkaloid was found to inhibit the growth of *Leishmania braziliensis* promastigotes (100% at 25 μM) and of *Trypanosoma cruzi* epimastigotes in culture (50% at 25 μM) and showed a strong in vitro antiplasmodial activity with an IC_{50} of 25 nM.

In the course of a screening program for potential antiprotozoal drugs from Bolivian plants, an ethanolic extract from the leaves of *Saracha punctata* Ruíz et Pavón was screened for its activity against different strains of *Leishmania* (responsible for leishmaniasis), against *Trypanosoma cruzi* (the causative factor of Chagas' disease), and against the rodent malaria parasites *Plasmodium vinckei* and *Plasmodium berghei*. The genus *Saracha* belongs to the Solaninae subtribe of the family Solanaceae and consists of three species growing in the Andes at an elevation of 2700−4000 m.[1] Distribution of *S. punctata*, the only species in this genus that grows in Bolivia, is limited to the humid highland forests named "Yungas", where it is rather common as a shrub.[2]

Until very recently, no previous chemical work had been published on the genus, and we describe the isolation and structure elucidation of a new steroidal amine (**1**) and the identification of two known flavonoids (**2** and **3**).[3] The leishmanicidal and antimalarial properties of the crude ethanolic extract of *S. punctata* and of alkaloid **1** will be briefly discussed.



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The ethanolic extract from the leaves of *S. punctata* was partitioned between *n*-butanol and water. The residue obtained after evaporation of the butanol was chromatographed on Si gel and yielded compounds **2** and **3**. The aqueous layer was made alkaline and extracted with *n*-butanol to yield, after evaporation, one major compound (**1**), which we propose the trivial name sarachine. Compounds **2** and **3** were identified as the known compounds eriodictyol[4,5] and 7-*O*-β-D-glucopyranosyl-eriodictyol[6] by comparison of their spectral data with published values.

The molecular peak of sarachine (**1**) observed at *m*/*z* 398 in the EIMS suggested that the molecule contained two nitrogen atoms, and a molecular formula of $C_{27}H_{46}N_2$ was proposed after examination of the ^{13}C NMR spectrum. The gross features of the ^{1}H and ^{13}C NMR spectra suggested the steroid-like nature of the skeleton.[7] With the mass spectral fragmentation of steroidal amines being well documented, it was possible to deduce that the prominent peak at *m*/*z* 98 was the result of a fragmentation between C-20 and C-22 of a 22,26-epiminocholestane and that the ion at *m*/*z* 56 belonged to an amino group at C-3.[8] The ^{1}H NMR spectrum of **1** showed two three-proton singlets at δ 0.70 and 1.00 ppm for the angular methyl groups CH_3-18 and CH_3-19; two three-proton doublets at δ 0.83 (J = 7.0 Hz) and 0.94 (J = 7.0 Hz), corresponding to the two secondary methyl groups CH_3-27 and CH_3-21; and one broad doublet at δ 5.32 (1H, J = 4.0 Hz) for the olefinic proton. These signals were those expected for a $\Delta^{5,6}$-cholestene steroidal amine.[7] Four protons were observed between 2.2 and 3.1 ppm, attributable to four α-amino protons, which were attached to two methine amino carbons and one methylene amino carbon resonating between 52 and 60 ppm (HMQC). Most ^{13}C NMR signals of **1** were assigned by analysis of HMQC and HMBC correlations, except methylenes C-2, C-11, C-15, C-16, and C-23, which were attributed by comparison with literature data.[9−11] In the HMBC spectrum, the doublet of CH_3-27 was correlated with the methylene C-26 carbon at δ 54.2; this carbon was linked to two coupled protons, one triplet at δ 2.28 (J = 11.5 Hz), and one broad doublet at δ 3.05 in the HMQC spectrum. The value of the ^3J coupling constant suggested that H-25 was in the axial position. The chemical shift of C-27 at δ 19.4 confirmed that the terminal methyl was in an equatorial position as in isoteinemine[12] and solafloridine.[13] The CH_3-21 doublet exhibited a long-range H−C correlation with C-22 at δ 59.4, which bears one proton and appeared as a doublet of multiplets at δ 2.49 (J = 11.0 Hz). The

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Table 1. In Vitro Antileishmanial Activity on Promastigote and Amastigote Forms of *Leishmania* spp. and Trypanocidal Activity on Epimastigote Forms of *Trypanosoma cruzi* of the EtOH Extract from *Saracha punctata* and Sarachine (**1**)

	EtOH extract (μg/mL)					sarachine (**1**) (μg/mL)					pentamidine (μg/mL)
	100	50	25	12.5	10	20	10	5	2.5	0.5	10
T. cruzi (% inhib.)	100	100	100	50		100	50	0	0		100
L. brasiliensis 2903	100	100	100	100		100	100	50	0		100
L. donovani chagasi PP75	100	100	100	50		100	100	50	0		100
L. amazonensis 142	100	100	100	50		100	100	50	0		100
amastigote survival					100						
macrophage survival	0	0	0	0	80	0	0	0	ND	95	

Table 2. In Vivo Antimalarial Activity of EtOH Extract from *Saracha punctata* and Sarachine (**1**)

strain	doses (mg/kg/*x* days)	% parasitemia on Day 4 (± S.E.M.)	% suppresion of parasitemia	lethality on Day 4
P. vinckei petteri (279 BY)				
	Control	73 ± 7		
	EtOH Extract			
	200 ($x = 2$ days)	1	96	8
	100 ($x = 2$ days)	4 ± 2	83	2
	50 ($x = 4$ days)	38 ± 12	48	5
P. berghei (NK65)				
	Control	88 ± 4		
	Sarachine (**1**)			
	32	27 ± 9	69	3
	16	64 ± 8	27	0
	8	74 ± 5	16	0

last α-amino proton, a triplet of triplets ($J = 11.0$, 4.5 Hz) at δ 2.60 corresponded to the axially oriented hydrogen at C-3. The observation of a ROE effect between CH_3-18 and H-20 was in agreement with the usual 17β side-chain configuration.

Detailed analysis of ROE and inter-proton coupling constants in the piperidine ring of **1** allowed the determination of the relative configurations of C-22 and C-25 but not their absolute configurations. The determination of the C-22 and C-25 configurations in 22,26-epiminosteroids has been an object of debate and controversy settled by a still unpublished X-ray crystallographic study and a partial synthesis.[11,14] Owing to the equatorial nature of CH_3-27 (δ_C 19 ppm vs 16 ppm), compound **1** does not have the side chain of desacetylmuldamine (or teinemine), nor does it have the configuration of isoteinemine.

Table 1 reports the in vitro activities of the crude alcoholic extract from the leaves of *S. punctata* as well as that of sarachine (**1**) on various strains of the promastigote forms of *Leishmania* spp. Complete inhibition of the promastigote culture by the crude extract occurred at a concentration of 12.5 μg/mL with the more sensitive strain (*Leishmania braziliensis* 2903) and at 25 μg/mL with the other strains. The crude extract did not display any activity for the intracellular amastigote form at 10 μg/mL and showed toxicity toward macrophage host cells at 12.5 μg/mL (100% mortality). Alkaloid **1** showed higher activity toward the promastigote form of *Leishmania* [100% inhibition at 10 μg/mL (25 μM) against *L. braziliensis* 2903 strain] and inhibited the growth of epismatigote forms of *T. cruzi* in culture [50% inhibition at 10 μg/mL (25 μM)]. It was highly toxic against macrophage cells (no survival).

Ethanolic *S. punctata* extracts also reduced the virulence of experimentally induced *P. vinckei* infections in mice (Table 2). Alkaloid **1** showed in vitro antiplasmodial activity with an IC_{50} of 0.01 μg/mL (25 nM) against a chloroquine-sensitive strain, and an IC_{50} of 0.07 μg/mL (176 nM) against INDO-resistant strains. The IC_{50} value for KB cells was 20 μg/mL (50 μM), thus giving cytotoxicity-to-activity ratios of 2000 and 286, respectively, for the two strains. These values should be compared with a ratio of 846 obtained for chloroquine chlorhydrate against the sensitive strain (IC_{50} of 0.08 μg/mL = 223 nM), and a ratio of 345 obtained against resistant strain (IC_{50} of 0.20 μg/mL, 557 nM). These results suggest that, despite its high cellular toxicity toward macrophages, compound **1** exhibits better selective toxicity against *Plasmodium* than against KB tumor cells. Compound **1** was also active in vivo against *Plasmodium vinckei*, with 83% inhibition of the parasitemia at 100 mg/kg/2 days (Table 2).

In conclusion, we have at hand an abundant source of a new steroid (**1**) with interesting preliminary biological activities. Work is in progress to understand its antimalarial mode of action and to secure a detemination of the C-22 configuration.

Experimental Section

General Experimental Procedures. Optical rotations were determined with a Perkin−Elmer 241 polarimeter. ^1H NMR and ^{13}C NMR spectra were recorded with a Bruker AC 300 at 300 and 75 MHz, respectively, in $CDCl_3$. Two-dimensional NMR experiments were performed using standard Bruker microprograms. EIMS were obtained with an Autospec VG mass spectrometer. Si gel 60 (Geduran, 70−230 mesh, Merck) was used for column chromatography. TLC was performed on precoated TLC plates with Si gel 60 (K_6F, Whatman).

Parasites. *Leishmania amazonensis* strain MHOM/GF/84/CAY H-142 was originally isolated in the French Guyana Institut Pasteur. *Leishmania braziliensis* strain MHOM/BR/75/M 2903 was obtained from IBBA, a WHO reference laboratory; identifications were controlled by isoenzyme analysis. *Trypanosoma cruzi* strain Tuluhuen was used. The strain was obtained from IBBA, and the identification was confirmed by isoenzyme analysis.

Plant Material. Samples from *S. punctata* were collected in the Bolivian "Yungas" at an elevation of 2700 m, in a place named "Siberia", 150 km from Cochabamba on the Santa Cruz road in August 1989. Herbarium specimens were identified and deposited in the U. M. S. A. National Herbarium of La Paz (voucher specimen Moretti 1458).

Extraction and Isolation. Dried ground leaves (0.7 kg) were successively extracted with petroleum ether and EtOH

in a Soxhlet apparatus. Evaporation of EtOH in vacuo gave a gum (220 g) that was dialyzed against pure H_2O. After freeze-drying, a part of the residue (54 g) was partitioned between H_2O and *n*-BuOH. Evaporation of the organic layer yielded a powder (25 g), 4.5 g of which were chromatographed over Si gel eluted with $CHCl_3$−MeOH (49:1) to give 140 mg of flavonoid **2** and with $CHCl_3$−MeOH (9:1) to give 460 mg of the flavonoid glycoside **3**. The aqueous layer was made alkaline with NH_4OH and extracted with *n*-BuOH. Evaporation of the organic layer yielded 3.4 g of steroidal amine **1** as a greenish powder.

3β-Amino-22,26-epiminocholest-5-ene (sarachine) (1): $[\alpha]^{25}_D$ −7°(*c* 0.3, $CHCl_3$); $[\alpha]^{25}_D$ −13°(*c* 0.3, MeOH); [chlorhydrate: $[\alpha]^{25}_D$ −21.6°(*c* 0.5, MeOH)]; ^1H NMR ($CDCl_3$, 300 MHz) δ 5.32 (1H, br d, *J* = 4.0 Hz, H-6), 3.05 (1H, m, H-26a), 2.60 (1H, tt, *J* = 11.0, 4.5 Hz, H-3), 2.49 (1H, dm, *J* = 11.0 Hz, H-22), 2.28 (1H, t, *J* = 11.5 Hz, H-26b), 2.15 (1H, ddd, *J* = 13.0, 4.5, 2.0 Hz, H-4a), 2.05 (1H, td, *J* = 12.0, 2.0 Hz, H-4b), 1.99 (1H, m, H-12a), 1.98 (2H, m, H-7), 1.80 (3H, m, H-1a, H-16a, H-24a), 1.68 (1H, m, H-8), 1.65−1.50 (4H, m, H-15, H-2), 1.60 (1H, m, H-20), 1.55−1.40 (2H, m, H-11), 1.45 (1H, m, H-25), 1.25−1.10 (2H, m, H-23),1.20 (1H, br d, *J* = 9.0 Hz, H-17), 1.12 (1H, m, H-12b), 1.08 (1H, m, H-1b), 1.00 (2H, m, H-14, H-24b), 1.00 (3H, s, H-19), 0.94 (3H, d, *J* = 7.0 Hz, H-21), 0.94 (1H, m, H-9), 0.83 (1H, m, H-16b), 0.83 (3H, d, *J* = 7.0 Hz, H-27), 0.70 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 141.8 (s, C-5), 120.6 (d, C-6), 59.4 (d, C-22), 56.6 (d, C-14), 54.2 (t, C-26), 53.0 (d, C-17), 52.0 (d, C-3), 50.2 (d, C-9), 43.2 (t, C-4), 42.4 (s, C-13), 40.1 (d, C-20), 39.9 (t, C-12), 38.2 (t, C-1), 36.5 (s, C-10), 33.4 (t, C-24), 32.6 (t, C-2), 31.9 (d, C-25), 31.8 (t, C-7), 31.6 (d, C-8), 27.7 (t, C-16), 24.4 (t, C-23), 24.2 (t, C-15), 21.0 (t, C-11), 19.4 (q, C-27), 19.3 (q, C-19), 13.6 (q, C-21), 11.8 (q, C-18); EIMS *m/z* $[M]^+$ 398 (42), 382 (15), 356 (13), 328 (10), 302 (26), 284 (26), 256 (33), 213 (25), 185 (18), 173 (24), 159 (30), 145 (32), 133 (39), 125 (99), 107 (99), 99 (100), 98 (100), 56 (65); HRFABMS *m/z* $[M + H]^+$ 399.3708 (calcd for $C_{27}H_{47}N_2$ 399.3739).

Eriodictyol (2): ^1H NMR ($CDCl_3$, 300 MHz) δ 6.91 (1H, br s, H-2′), 6.78 (2H, m, H-6′ and H-8′), 5.90 (1H, d, *J* = 2.1 Hz, H-6), 5.88 (1H, d, *J* = 2.1 Hz, H-8), 5.27 (1H, dd, *J* = 12.7, 3 Hz, H-2), 3.06 (1H, dd, *J* = 17.2, 12.7 Hz, H-3ax), 2.68 (1H, dd, *J* = 17.2, 3 Hz, H-3eq); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 197.8 (C-4), 168.4 (C-7), 163.0 (C-5), 164.9 (C-8a), 148.0 (C-3′), 147.0 (C-4′), 131.8 (C-1′), 119.3 (C-6′), 116.3 (C-5′), 114.7 (C-2′), 104 (C-4a), 97.0 (C-8), 96.2 (C-6), 80.5 (C-2), 44.1 (C-3); EIMS *m/z* 288 (85), 179 (30), 166 (45), 153 (100),136 (50).

7-*O*-β-D-Glucopyranosyl-eriodictyol (3): ^1H NMR (CD_3OD, 300 MHz) δ 6.91 (1H, br s, H-2′), 6.78 (2H, br s, H-6′ and H-8′), 6.20 (1H, d, *J* = 2.0 Hz, H-6), 6.18 (1H, d, *J* = 2.0 Hz, H-8), 5.30 (1H, dd, *J* = 12.0, 3.0 Hz, H-2), 4.90 (1H, d, *J* = 7.0 Hz, Glc-1), 3.88 (1H, br d, *J* = 12.0 Hz, Glc-6a), 3.66 (1H, dd, *J* = 12.0, 5.0 Hz, Glc-6b), 3.45 (4H, m, Glc-2, Glc-3, Glc-4, Glc-5), 3.11 (1H, dd, *J* = 16.0, 12.0 Hz, H-3ax), 2.72 (1H, dd, *J* = 16.0, 3.0 Hz, H-3eq); ^{13}C NMR (CD_3OD, 75 MHz) δ 198.5 (C-4), 167.0 (C-7), 164.9 (C-5), 164.5 (C-8a), 146.9 (C-3′), 146.5 (C-4′), 131.4 (C-1′), 119.3 (C-6′), 116.2 (C-5′), 114.8 (C-2′), 104.9 (C-4a), 101.1 (Glc-1), 97.9 (C-8), 96.8 (C-6), 80.6 (C-2), 78.2 (Glc-3), 77.7 (Glc-5), 74.6 (Glc-2), 71.1 (Glc-4), 62.3 (Glc-6), 44.2 (C-3).

Leishmanicidal Activity. In vitro test procedure on promastigote culture of *Leishmania* spp.: Compounds were aseptically dissolved in liquid medium and DMSO (final concentration of DMSO less than 0.1%) and placed in microcells Titertek 96 (Flow Laboratories) to obtain final concentrations of 100, 50, 25, and 12.5 μg/mL. All assays were done in triplicate. Each cell was cultured with 50 000 parasites at 27 °C. The activity of the compounds was evaluated after 72 h by optical observation on a drop of culture with an inverted-phase microscope, by comparison with control cells without extracts.[15]

In vitro test procedure on the amastigote forms of *Leishmania*: Mouse peritoneal macrophages were obtained according to a described procedure.[16] One million noninflammatory macrophages were collected from each BALB/c mouse. The adherent cells were cultured at 37 °C under 5% CO_2 over 2 h, then the plates were washed with RPMI+buffer (MOPS−Sigma, USA), without fetal calf serum (FCS), to eliminate nonadherent cells. The supernatant was replaced by 0.5 mL/well of fresh medium RPMI+glutamine+FCS+ antibiotics before infection by *L. amazonensis* amastigotes at a ratio of infecting organism to host cell of 5:1. Infection took place at 34 °C over a minimum of 2 h, and the compounds were added to the culture maintained at 37 °C under 5% CO_2 for 24 h. The medium was then renewed, and the cells left to incubate for another 24 h before fixation. Plates were fixed with MeOH and stained with 10% Giemsa's stain (Specia, France). They were set up with Eukitt Resin (CML, France). Macrophages with and without parasites were counted under 40× magnification. For each triplicate assay, the survival index (SI) of amastigotes was calculated relative to the control.

Determination of Trypanocidal Activity. In vitro procedure on the epimastigote form of *T. cruzi*: *T. cruzi* epimastigotes were cultured in liver infusion tryptose (LIT) medium supplemented with 10% FCS at 28 °C with an inoculum of 10^6 cells/mL. Samples (4 mg) were aseptically dissolved in 50 μL DMSO and liquid medium to obtain final concentrations of 20, 10, 5, 3, 1.5, and 0.75 μg/mL. All assays were carried out in triplicate. Final DMSO concentration was less than 0.5%. Parasites were counted after 48 h of contact with the samples in a hemocytometer, and the activity of the test substances was assessed by comparison with controls without extract.[17]

Determination of Antimalarial Activity. In vitro testing against *P. falciparum* was carried out using a method based on that of Desjardins et al.[18] Cultures of *P. falciparum* (chloroquine-sensitive strain 2087 and chloroquine-resistant strain INDO) were maintained in human erythrocytes according to reference.[19] DMSO (50 μL) was added to samples of extracts or pure compounds, which were then dissolved in RPMI 1640 medium with the aid of mild sonication in a sonicleaner bath (Branson Ltd.) and further diluted as required in medium. The DMSO concentration for tested dilutions was no greater than 0.1%. The total culture medium (150 μL) was placed into the wells of 96-well microtiter plates with the diluted extract and the suspension of human red blood cells in medium (0^+, 5% hematocrit) with 1% parasitemia. All tests were performed in triplicate. After 24 h of incubation at 37 °C using the candle-jar method, the medium was replaced fresh daily, and incubation was continued for a further 48 h. On the third day of the test, a blood smear was taken from each well, and parasitemia counted. Each test included an untreated control, control with solvent, and chloroquine as an internal standard. The parasitemia for each well was obtained, and the % inhibition of parasitemia for each concentration of extracts was calculated in relation to the control. Linear regression analysis was used to determine the best fitting straight line from which IC_{50} values were determined.

In vivo testing against *P. vinckei* and *P. berghei*: The four-day suppressive test, adapted from Peters, against *P. vinckei* and *P. berghei* infection in mice was used.[20−22] Mice were inoculated with *P. berghei* NK 65 or *P. vinckei petteri* 279BY on day 1 of the experiment and inoculated daily for four consecutive days with the extract or drug under test. On day 5 of the test, a blood smear was taken. ED_{50} values were computed by comparing the parasitemias present in infected controls with those of test animals.

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