New Leishmanicidal Stilbenes from a Peruvian Folk Medicine, *Lonchocarpus nicou*

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Two new stilbenes, 1 and 2, were isolated as leishmanicidal constituents from the methanolic extract of *Lonchocarpus nicou* leaves and stem, together with five known stilbenes and rotenoids. Their chemical structures were determined by spectral methods. Among them, the *cis* stilbene-type compounds 1 and 4 showed potent leishmanicidal activity (IC_{50}: 5.5, 3.9 μg/mL), while the *trans* stilbene-type compounds 2 and 5, and rotenoids 6 and 7, showed moderate activities (IC_{50}: 9.9, 12.8, 22.6, 19.6 μg/mL, respectively).

Key words Barbasco; *Lonchocarpus nicou*; Leishmania; Leguminosae; Peruvian folk medicine

Peru has both Andean and Amazon regions, which show quite different vegetations. While many plants are indigenous to the Amazon region, there have been few chemical and pharmacological studies of the plants in this area. Thus, Peruvian plants represent a fascinating resource for drug discovery.

Leishmaniasis is endemic in tropical regions. Leishmaniasis currently affects 12 million people in 88 countries.[1)] The disease is transmitted by small biting sandflies (*Phlebotomus* spp.). The first-line drugs for the treatment of leishmaniasis are pentavalent antimonials such as *N*-methylglucamine antimonate (Glucantime) and sodium stilbogluconate (Pentostam); however, these drugs are toxic and generally expensive.

The Peruvian Leguminosae plant *Lonchocarpus nicou* DC. (local name: Barbasco) (Leguminosae) is a local plant in the Amazon region and is well known to contain rotenone, which shows strong insecticidal activity. Leaves from this plant have been used as a poultice for the treatment of cutaneous leishmaniasis, and a decoction of the leaves or root has also been used to clean affected areas. A decoction of the root is also used to treat skin diseases, and stem juice is used to treat mycotic disease. It is said that this plant is effective for malaria and three-day fever.[2)] Although several rotenoids, stilbenes and two *C*-prenylated benzenes have been obtained from *L. nicou* root,[3–7)] the constituents of the leaves and stem have not been previously reported.

In a preliminary study, we screened several Peruvian folk medicines for anti-leishmanial activity, and found many active compounds.[8–13)] In this screening, we found that the methanolic extract of *Lonchocarpus nicou* showed potent activity. Therefore, we investigated the active components of this plant.

In this paper, we describe the leishmanicidal constituents of Barbasco.

Experimental

General Experimental Procedures ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL Alpha 500 spectrometer (^{1}H: 500 MHz). HPLC was performed on a Shimadzu LC-10ADVP system. LC-MS was measured on a QSTAR XL (AB Sciex) with turboion spray (TIS) and an atmospheric pressure chemical ionization (APCI) ion source.

Materials *L. nicou* was collected in Peru and identified by Dr. Elsa Renjifo (Peruvian Amazon Research Institute, Iquitos, Peru) and Dr. Juan Ruiz (National University of the Peruvian Amazon, Iquitos, Peru). The methanol extract used in this research was prepared by Dr. Victor Zorrilla (Institute of Tropical Medicine, Lima, Peru) and Mrs. Diana Flores (Latin Pharma, Peru). A voucher specimen (collection No. 2715) was deposited at the Peruvian Amazon Research Institute.

Chemicals Column chromatography fractions were analyzed on Merck TLC glass plates (No. 105715). Rotenone was purchased from MP Biomedicals, LLC. Tetra Color ONE was purchased from Seikagaku Kogyo Co., Ltd. Medium 199 was obtained from Invitrogen Co., Ltd. ES Cell Qualified Fetal Bovine Serum was purchased from Invitrogen Co., Ltd.

Cultivation of Leishmania Promastigotes Medium 199 was used to cultivate promastigotes of *Leishmania major* (MHOM/SU/73/5ASKH). Promastigotes were cultured in the medium supplemented with heat-inactivated (56°C for 30 min) fetal bovine serum (10%) at 27°C, 5% CO_2 in an incubator.

Leishmanicidal Activity Assay The leishmanicidal effects of extracts and isolated compounds were assessed by the improved 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasodium bromide (MTT) method, as follows. Cultured promastigotes were seeded at 2×10^4/50 μL of medium per well in 96-well microplates, and then 50 μL of different concentrations of test compounds dissolved in a mixture of dimethyl sulfoxide (DMSO) and the medium were added to each well. Each concentration was tested in triplicate. The

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microplate was incubated at 27°C in 5% CO_2 for 48 h. Tetra Color ONE (10 μL) (a mixture of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt (WST-8) and 1-methoxy-5-methylphenazinium methosulfate (PMS)) was added to each well and the plates were incubated at 27°C for 6 h. Optical density values (test wavelength 450 nm; reference wavelength 630 nm) were measured using a microplate reader (Sunrise™, Tecan, Switzerland). The inhibitory concentration of 50% (IC_{50}) for isolated compounds was estimated from a graph.

Isolation Air-dried leaves (2.0 kg) and stems (1.12 kg) were extracted with methanol at 50°C for 4 h. After the extracts were filtered, the filtrates were concentrated to give brownish residues. From methanolic extracts of the leaves and stems, 280 g (leaves) and 56 g (stems) of residue were obtained, respectively. The extract of leaves showed a moderate leishmanicidal effect (MLC 100 μg/mL). The leaf extract (25.5 g) was passed over activated charcoal and eluted with methanol (10 L), 30% chloroform/methanol (10 L) and chloroform (5 L) to give 3 fractions (M, C-L and C). Each fraction was concentrated under reduced pressure to give a syrup. The fraction eluted by 30% chloroform/methanol (C–M) was chromatographed on silica-gel using chloroform–methanol as an eluent to afford 22 fractions. Fractions eluted with 20% methanol/chloroform were combined and subjected to preparative TLC (*n*-hexane–ethyl acetate=4 : 1) to give **1** (30.9 mg). The fraction eluted with chloroform (C) was subjected to column chromatography on silica-gel using *n*-hexane–ethyl acetate as an eluent to give 30 fractions. Fractions eluted with 40% ethyl acetate/*n*-hexane were combined and concentrated, and then subjected to HPLC separation (column; Waters XBridge Prep C18, 10×250 mm, solvent system; acetonitrile–water, gradient program; 0–10 min 50% acetonitrile/water, 10–30 min 50–60% acetonitrile/water, 30–50 min 60–90% acetonitrile/water, flow rate; 3.0 mL/min, column temperature; 30°C, detection; UV 254 nm) to give **2** (83 mg) and selinone (6.1 mg).

The methanolic extract of the stem was partitioned between water and diethylether, and the ether layer was concentrated *in vacuo* to give a syrup. The syrup was subjected to medium pressure liquid-chromatography (MPLC) (column: Yamazen, Si-40-CL, 37 mm×150 mm, detector: UV 254 nm) using *n*-hexane–ethylacetate as an eluent to give 55 fractions. From fractions eluted with 10% ethylacetate/*n*-hexane, 3,5-dimethoxy-4′-hydroxy-3′-prenyl-*trans*-stilbene (**3**, 22 mg) and 3,5-dimethoxy-4′-*O*-prenyl-*cis*-stilbene (**5**, 87 mg) were obtained. Fractions eluted with 15% ethylacetate/*n*-hexane were concentrated and then rechromatographed by MPLC (column: Yamazen, Si-40A, 11 mm×300 mm, detector: UV 254 nm) using *n*-hexane–chloroform (2 : 1) to give **1** (5 mg) and 3,5-dimethoxy-4′-hydroxy-3′-prenyl-*trans*-stilbene (**3**, 2 mg). Other fractions eluted with 10% ethylacetate/*n*-hexane in the first MPLC separation were concentrated, and then rechromatographed on MPLC (column: Yamazen, Si-40A, 11 mm×300 mm, detector: UV 254 nm) with *n*-hexane–chloroform (2 : 1–1 : 1) to afford **2** (21 mg). Fractions eluted with 20% ethylacetate/*n*-hexane in the first MPLC were concentrated and then subjected to MPLC (column: Yamazen, Si-40A, 11 mm×300 mm, detector: 254 nm) with *n*-hexane–chloroform (1 : 1) and HPLC (Column: Waters X Bridge prep C18, 5 μm, solvent system: 90% acetonitrile, flow rate: 2.0 mL/min, detector: UV 254 nm) to give hydroxyrotenone (**6**, 6 mg) and hydroxydeguelin (**7**, 2 mg). Compound **1**: a colorless amorphous powder, HR-APCI-time-of-flight (TOF)-MS *m/z* 311.1632 ($[M+H]^+$) (Calcd for $C_{20}H_{23}O_3$: 311.1642), ^{1}H-, ^{13}C-NMR data are listed in Table 1. Compound **2**: a colorless amorphous powder, HR-APCI-TOF-MS *m/z* 311.1635 ($[M+H]^+$) (Calcd for $C_{20}H_{23}O_3$: 311.1642), ^{1}H-, ^{13}C-NMR data are listed in Table 1.

Results and Discussion

From the methanolic extract of leaves, two new compounds, **1** and **2**, were isolated together with a known flavanone, selinone.[14] On the other hand, from the stem extract, **2**–**7** were

Table 1. NMR Data for **1** and **2**

	1		**2**	
	^{13}C	^{1}H	^{13}C	^{1}H
1	139.8		140.0	
2	106.7	6.39 (brs)	104.7	6.53 (brs)
3	160.8		161.0	
4	100.5	6.25 (t, *J*=2.4 Hz)	100.6	6.24 (t, *J*=2.4 Hz)
5	156.5		156.8	
6	108.2	6.32 (brs)	105.7	6.49 (brs)
7	130.2	6.38 (d, *J*=12.2 Hz)	126.1	6.89 (d, *J*=16.6 Hz)
8	128.3	6.48 (d, *J*=12.2 Hz)	128.9	6.76 (d, *J*=16.6 Hz)
1′	129.4		129.8	
2′	130.2	7.18 (d, *J*=8.8 Hz)	127.8	7.32 (d, *J*=8.8 Hz)
3′	114.3	6.76 (d, *J*=8.8 Hz)	114.9	6.81 (d, *J*=8.8 Hz)
4′	158.0		158.7	
5′	114.3	6.76 (d, *J*=8.8 Hz)	114.9	6.81 (d, *J*=8.8 Hz)
6′	130.2	7.18 (d, *J*=8.8 Hz)	127.8	7.32 (d, *J*=8.8 Hz)
1″	64.7	4.46 (d, *J*=6.8 Hz)	64.9	4.43 (d, *J*=6.8 Hz)
2″	119.6	5.45 (t, *J*=6.8 Hz)	119.5	5.42 (t, *J*=6.8 Hz)
3″	138.2		138.4	
4″	25.8	1.77 (s)	25.8	1.71 (s)
5″	18.2	1.71 (s)	18.2	1.66 (s)
OCH_3	55.2	3.64 (s)	55.3	3.71 (s)

isolated by activity-guided fractionation.

From the leaf extract, **1** and **2** were isolated as described in Experimental. Their chemical structures were elucidated as follows.

Compound **1** was assigned a formula of $C_{20}H_{22}O_3$ by APCI-TOF-MS and showed strong UV absorption on TLC. The ^{13}C-NMR spectrum showed 16 sp^2 carbons, two methyl groups (δ: 18.2, 25.8 ppm), a methylene group (δ: 64.7 ppm) and a methoxyl group on an aromatic ring (δ: 55.2 ppm). In the ^{1}H-NMR spectrum, the coupling constants of low-field signals indicated the presence of a *p*-substituted (δ: 7.18, 2H, d, J=8.8 Hz; 6.76, 2H, d, J=8.8 Hz) benzene ring and a *cis* olefin (δ: 6.38, 1H, d, J=12.2 Hz; 6.48, 1H, d, J=12.2 Hz). Furthermore, the presence of a 1,3,5-trisubstituted (δ: 6.25, 1H, t, J=2.4 Hz; 6.39, 1H, brs; 6.32, 1H, brs) aromatic ring system and a prenyloxyl group (δ: 5.45, 1H, t, J=6.8 Hz; 1.77, 3H, s; 1.71, 3H, s; 4.46, 2H, d, J=6.8 Hz) was deduced. Based on this information, **1** was thought to have a *cis*-stilbene skeleton. The nuclear Overhauser effect (NOE) correlation between the methoxyl group and two aromatic protons (δ: 6.32, 6.39) indicated that the methoxyl group is on the 1,3,5-trisubstituted aromatic ring. Thus, the chemical structure of **1** was deduced to be as shown in Fig. 1. The results of 2D-NMR (double quantum filtered-correlation spectroscopy (DQF-COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC)) supported this structure.

Compound **2** was assigned a formula of $C_{20}H_{22}O_3$ by high-resolution TOF-MS. The ^{1}H- and ^{13}C-NMR spectra were similar to those of **1** except for olefinic signals. In the ^{1}H-NMR spectrum, two doublet signals (δ: 6.84, 6.98 ppm) were observed and their coupling constants were 16.6 Hz. Therefore, **2** was deduced to contain a *trans* olefin. Based on the 2D-NMR correlations, the chemical structure of **2** was determined to be the *trans* isomer of **1**.

Other isolated leishmanicidal compounds were determined to be 3,5-dimethoxy-4′-hydroxy-3′-prenyl-*trans*-stilbene (**3**),[15] 3,5-dimethoxy-4′-*O*-prenyl-*cis*-stilbene (**4**),[16] 3,5-dimethoxy-4′-*O*-prenyl-*trans*-stilbene (**5**),[16] rotenolone (12-hydroxyrotenone) (**6**)[17] and tephrosin (**7**),[18] as shown in Fig. 2, based on a comparison of their spectra to those in the literature.

The leishmanicidal activities (IC_{50}) of compounds **1**–**7** were in the range of 3.9–40 μg/mL (Table 2). Among them, **4** showed the most potent activity (3.9 μg/mL), and **1** also showed potent activity (5.5 μg/mL). Compounds **5** and **2**, which are *trans* isomers of **4** and **1**, showed moderate activity (12.8, 9.9 μg/mL, respectively). Rotenone-type compounds **6** and **7** showed moderate activity (22.6, 19.6 μg/mL). Interestingly, **3**, which is an isomer of **5** in the position of prenyl group, showed the weakest activity.

In summary, a stilbene skeleton showed more potent activity than a rotenone skeleton, and *cis* isomers were more potent

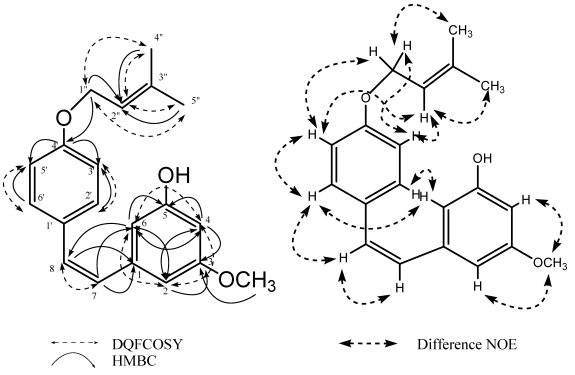


Fig. 1. 2D-NMR Correlations for **1**

Table 2. Leishmanicidal Activities for **1**–**7**

Compound	IC_{50} (μg/mL)
1	5.5
2	9.9
3	39.9
4	3.9
5	12.8
6	22.6
7	19.6
Rotenone	4.0

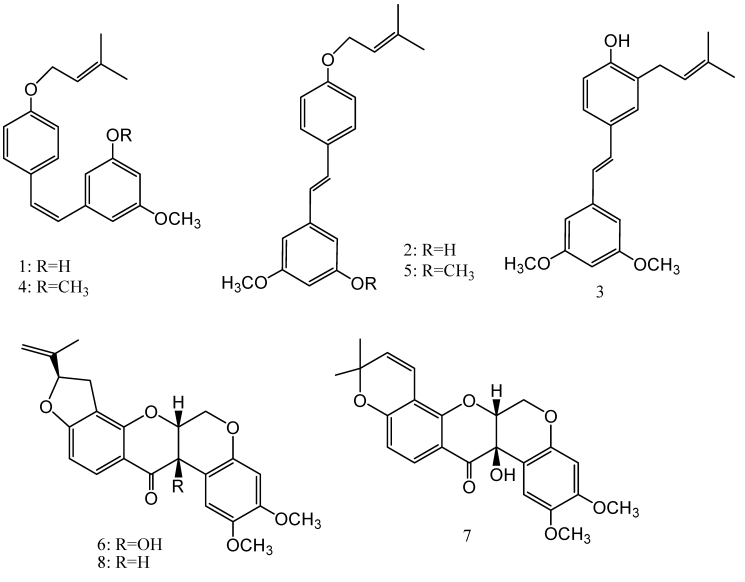


Fig. 2. Chemical Structures of Isolated Compounds

than the respective *trans* isomers. Rotenone is a well known fish poison, and is a main constituent of Barbasco root. However, we did not find it in the leaves or stems. Leishmanicidal activity of rotenone was examined using a commercial reagent (IC_{50} 4.0 μg/mL). The differences in activity between *cis* and *trans* isomers might be due to steric hindrance, although a mechanistic study will be needed to confirm this in the near future.

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