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Antitrypanosomal activity of a novel norlignan purified from *Nectandra lineata*

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Bioactivity-guided fractionation of a MeOH–EtOAc extract from the young leaves of *Nectandra lineata* (Lauraceae), using a *Trypanosoma cruzi* bioassay resulted in the isolation of a novel norlignan 3'-methoxy-3,4-methylenedioxy-4',7-epoxy-9-nor-8,5'-neolignan-9'-acetoxy (**1**), together with the known compound, 3'-methoxy-3,4-methylenedioxy-4'-7-epoxy-9-nor-8,5'-neolignan-7,8'-diene (**2**). Compounds **1** and **2** were shown to inhibit the growth of *T. cruzi* epimastigotes in axenic culture.

Keywords: *Trypanosoma cruzi*; Norlignan; *Nectandra lineata*; Lauraceae; Chagas' disease; ICBG

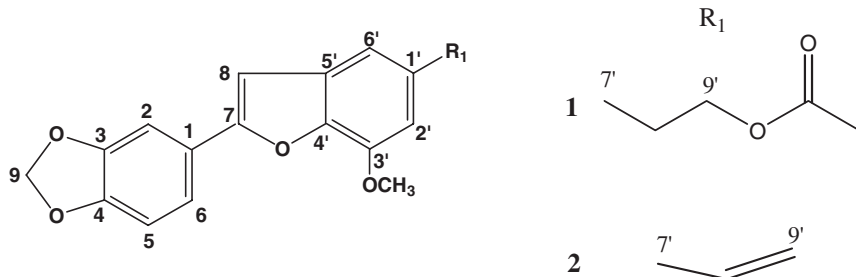
1. Introduction

Estimates from the World Bank rank Chagas' disease as the third most important cause of disability attributable to parasitic diseases, after malaria and schistosomiasis [1]. Around 16–18 million people in Latin America are infected with *Trypanosoma cruzi*, the parasite that causes Chagas' disease, and more than 100 million people are exposed to the risk of infection [2]. As part of the International Cooperative Biodiversity Groups (ICBG) Program established in the Republic of Panama, we are carrying out a systematic study of extracts from young and mature leaves of a terrestrial plant species collected in protected areas of Panamanian rainforests for activity against malaria, leishmaniasis, Chagas' disease, cancer and HIV [3,4].

We describe herein the bioassay-guided isolation of two norlignans (**1** and **2**) from *Nectandra lineata* (Family: Lauraceae) that were shown to inhibit the growth of *T. cruzi* epimastigotes in axenic culture, one of which (**1**) represents a novel compound. The norlignans show a range of activities including antibacterial and antifungal

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properties [5], as facilitators of adrenaline-mediated vascular contractions [6], and as inhibitors of hyaluronidase [7].



2. Results and discussion

An MeOH–EtOAc extract from leaves of *N. lineata* was highly active in *T. cruzi* bioassays ($IC_{50} = 5 \mu\text{g/mL}$). The crude extract was subjected to liquid–liquid partitioning with hexane, EtOAc, MeOH and H₂O. The hexane-soluble fraction showed the greatest activity against *T. cruzi* and was subjected to silica gel chromatography yielding norlignans **1** and **2**.

Compound **1** was obtained as a white amorphous powder, and HRFABMS data yielded a molecular formula of C₂₁H₂₀O₆. The structure of **1** was determined by NMR analysis, including HMQC and HMBC experiments, which enabled the assignment of all protons and carbons. The IR spectrum showed absorptions at 3000 and 1733 cm⁻¹. Of the 21 signals in the ¹³C NMR spectrum, two were methyls, four were methylenes and six were methines. The ¹H NMR spectrum showed an acetate methyl (δ 2.09), a methoxy group (δ 4.05), an oxymethylene (δ 4.14), a methylenedioxy group (δ 6.02) and a singlet aromatic proton signal at δ 6.81 (H-8). The remaining aromatic ¹H signals could be ascribed to H-6 (δ 7.42, dd, $J = 1.7$ and 8.1 Hz), H-2 (δ 7.34, d, $J = 1.7$ Hz) and H-5 (δ 6.88, d, $J = 8.1$ Hz) in one benzene ring, and H-2' (δ 6.63, d, $J = 1.5$ Hz) and H-6' (δ 6.96, d, $J = 1.5$ Hz) in a second benzene ring. The signal at δ 6.63 (H-2') was correlated in the HMBC spectrum with C-1', C-3', C-4' and C-6', while a correlation from the singlet methyl at δ 4.05 to C-3' (δ 144.8) placed the methoxy substituent. The fused furan moiety was determined by the strong correlations from H-8 to the quaternary carbons at δ 156.2 (C-7), δ 142.6 (C-4'), δ 131.1 (C-5') and a weak correlation to δ 144.8 (C-3') and δ 112.3 (C-6'), while the connectivity of the two fused cyclic motifs was defined by weak three-bond correlations from H-6 to C-7. H-6 (δ 7.42) also showed correlations to δ 105.5 (C-2), δ 148.0/148.1 (C-3/4), which, together with correlations from the methylenedioxy to C-3/4, established the benzyl-dioxan portion of the molecule.

The NMR data for compounds **1** and **2** were very similar. In the ¹H NMR spectrum of **2**, the acetyl group and two methylenes were absent, while an olefinic proton (δ 5.95–6.05, 1H, m) and two exomethylene proton signals (δ 4.49–5.10, 2H, m) were apparent. Furthermore, the carbonyl carbon and acetate methyl in **1** were absent in the spectrum of **2**. We also noticed the substitution of a propylacetate group at C-1'

in **1** for an allyl group at C-1' in **2**. The isolation of compound **2** from *Anaxagorea clavata* (Annonaceae) was previously described by Puentes De Díaz [8]. After a detailed analysis of the HMQC and HMBC data, not reported in published work, we have reassigned the δ 156.2 (C-3'), δ 144.8 (C-4') and δ 142.6 (C-7) to carbons C-7, C-3' and C-4' respectively, for the structure originally isolated by Puentes De Díaz.

Compound **2** showed good activity against *T. cruzi* with an IC_{50} = 60 μ M. On the contrary, compound **1** had a lower measured activity against *T. cruzi* (IC_{50} = 111 μ M). The lower observed anti-trypanosomal activity of **1** is likely to be due to its incomplete solubility under the conditions of our bioassay, despite the attempts to enhance the solubility with a variety of different co-solvents such as Tween-80, glycerol and polyamide.

3. Experimental section

3.1. General experimental procedures

Melting points were determined using an Electrothermal 9100 apparatus and are uncorrected. IR spectra were measured on a Shimadzu FTIR-8300 spectrophotometer. 1H NMR data were recorded on a Bruker Avance 300 MHz spectrometer (^{13}C NMR at 75 MHz). Low-resolution EIMS (70 eV) data were collected on a JEOL SX 102A mass spectrometer and HRMS on a Kratos MS50TC instrument.

3.2. Plant material

Young leaves of *N. lineata* were collected from Barro Colorado Nature Monument in Gatún Lake, in January 2001. The material was identified by both Professor Mireya Correa of the Smithsonian Tropical Research Institute and the University of Panamá. Voucher specimens (PMA 50975) of *N. lineata* have been deposited in the herbarium of the University of Panamá. Young leaves were selected as they often contain higher levels of secondary metabolites than mature leaves of the same species [3].

3.3. Extraction and isolation

Upon collection, leaves were transferred to sealed plastic bags, kept on ice and processed within 6 h. After removal of the stems, 0.9 kg of fresh leaves were homogenized in 30 g aliquots with 240 mL of cold methanol in a Waring blender followed by treatment with a Polytron homogenizer (Brinkmann Instruments, Inc.) for 2 min or until the suspension of leaf material was homogeneous. The mixture was filtered under vacuum through Whatman #4 filter paper and the residue was then washed with EtOAc (150 mL). The MeOH and EtOAc fractions were combined and filtered through Whatman #1 filter paper. The extract was concentrated by rotary evaporation and stored at $-80^\circ C$ until further use.

For bioassay-guided purification, the crude extract was partitioned between H_2O and CH_2Cl_2 . The water soluble partition was then extracted with EtOAc, while the CH_2Cl_2 partition was evaporated and then partitioned between hexane and 90% MeOH. The hexane soluble fraction was subjected to silica gel (230–400 mesh)

column chromatography eluting with EtOAc:hexane (3:7) yielding fractions 1a–21a. Fraction 2a (290 mg) was purified by crystallization from MeOH to give compound **2** (3'-methoxy-3,4-methylenedioxy-4'-7-epoxy-9-nor-8,5'-neolignan-7,8'-diene) [8]. Fraction 6a was further chromatographed on silica gel eluting with hexane:CH₂Cl₂, 4:6 yielding fractions 1b–15b. From fractions 10b (70 mg) and 12b (268 mg), compound **1** (57 mg) was crystallized from MeOH.

3.3.1. 3'-Methoxy-3,4-methylenedioxy-4',7-epoxy-9-nor-8,5'-neolignan-9'-acetoxy (**1**).

White solid, m.p. 127–130°C, IR (film): 3000, 1733, 1477, 1363 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ 7.42 (dd, H-6, *J* = 1.7 and 8.1 Hz), 7.34 (d, H-2, *J* = 1.7 Hz), 6.97 (d, H-6', *J* = 1.4 Hz), 6.88 (d, H-5, *J* = 8.1 Hz), 6.81 (s, H-8), 6.63 (d, H-2', *J* = 1.4 Hz), 6.02 (s, H-9), 4.14 (t, H-9', *J* = 6.6 Hz), 4.05 (s, H-12'), 2.77 (t, H-7', *J* = 7.6 Hz), 2.09 (s, H-11'), 2.02 (m, H-8'); ¹³C NMR (75 MHz, CDCl₃) δ 171.0 (C-10'), 156.2 (C-7), 148.1 (C-4), 148.0 (C-3), 144.8 (C-3'), 142.6 (C-4'), 137.5 (C-8'), 135.7 (C-1'), 131.1 (C-5'), 124.7 (C-1), 119.2 (C-6), 115.6 (C-9'), 112.3 (C-6'), 108.6 (C-5), 107.4 (C-2'), 105.6 (C-2), 101.3 (OCH₂O), 100.4 (C-8), 63.9 (C-9'), 56.2 (MeO), 32.5 (C-7'), 30.7 (C-8'), 21.0 (CH₃CO); HRFABMS (NBA, positive mode) M⁺ 368.12540 (calcd for C₂₁H₂₀O₆, 368.12599); EIMS *m/z* 368 ([M]⁺, 43), 164 (14), 163 (100).

3.4. In vitro test for antitrypanosomal activity

The recombinant Tulahuen clone C4 of *T. cruzi* that expresses β-galactosidase (βGal) as a reporter enzyme was used in the assay [9]. Recombinant epimastigotes were grown in liver infusion tryptone (LIT) medium supplemented with 10% calf serum at 28°C as previously described [10]. Cultures were initiated with a cell density of 1 × 10⁵ epimastigotes per mL and were incubated in the presence of test compounds. Three days after seeding of the culture with parasite, the assays were developed by addition of the substrate, chlorophenol red-β-D-galactopyranoside (CPRG), to a final concentration of 100 μM and Nonidet P-40 to a final concentration of 0.1%. Plates were incubated for 4–6 h at 37°C, and the colorimetric reaction quantified with a Benchmark microplate reader (BIORAD) at an optical density of 570 nm. Negative controls contained DMSO at a final concentration identical to those employed to solubilize test substances. The absorbance observed at 570 nm from the negative control was subtracted from the IC₅₀ value determined in the presence of test substance. Each test substance as well as control experiment was tested in duplicate and the results represent a minimum of three separate experiments, which were used to calculate the standard deviation (SD). Results are expressed as IC₅₀, the concentration of compound that inhibited growth of the parasites by fifty percent ±SD. Nifurtimox and Amphotericin B, drugs known to have activity against *T. cruzi*, were used as positive controls for each assay [9].

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