Niruriside, a New HIV REV/RRE Binding Inhibitor from *Phyllanthus niruri*

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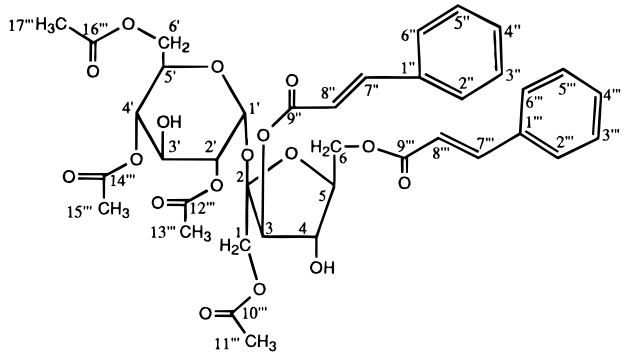
During the screening of natural products for their ability to inhibit the binding of HIV-REV protein to [^{33}P]-labeled RRE RNA, one novel compound, niruriside (**1**), was isolated from the MeOH extract of the dried leaf of *Phyllanthus niruri* L. by bioassay-guided fractionation. The structure of niruriside was determined by spectroscopic methods. Niruriside showed specific inhibitory activity against the binding of REV protein to RRE RNA with an IC_{50} value of 3.3 μM; however, niruriside did not protect CEM-SS cells from acute HIV infection at concentrations up to 260 μM using an XTT dye reduction assay.

Replication of human immunodeficiency virus (HIV) depends on many specific interactions between viral RNAs and proteins of viral and cellular origin. REV (regulation of virion expression) is an HIV protein that regulates the transport of viral RNA to the cytoplasm; thus, it is essential for HIV productive infection. The interaction of the basic domain of REV with the REV-responsive element (RRE), a stem-loop RNA structure in the envelope region, is required for REV function.[1−3] Inhibitors of this interaction could be of use as antiviral agents. Two classes of compounds have been described as such inhibitors: neomycin B, an aminoglycoside antibiotic,[4] and pyronin Y, an RNA intercalating agent.[5] In our study we developed a high-throughput screen to identify inhibitors of the formation of this REV/RRE complex. The specificity of the REV/RRE inhibitory activity was evaluated by an unrelated RNA/protein-binding assay, the R17 coat protein/R17 RNA system. Activity of the REV-specific compounds against HIV was studied with a cell protection (XTT dye reduction) assay. During the screening of natural products for their ability to inhibit the binding of REV protein to RRE RNA, the MeOH extract of the leaves of the Indian medicinal plant, *Phyllanthus niruri* L. (Euphorbiaceae), was found to be active.

P. niruri has been widely used against jaundice in Indian traditional medicine.[6] Various classes of chemical constituents, such as lignans, flavonoids, triterpenoids, tannins, and alkaloids, were isolated from this plant.[7−12] Its aqueous extract has been reported to inhibit DNA polymerase of hepatitis B and woodchuck hepatitis viruses,[13] the avian myeloblastosis virus reverse transcriptase (AMV-RT),[14] and human immunodeficiency virus type-1 reverse transcriptase (HIV-1-RT).[15] During our study, we isolated a new compound, designated as niruriside, by using bioassay-guided fractionation. In this report we describe the isolation, structure determination, specific inhibition of REV protein/RRE RNA binding, and other biological activities of niruriside.

From the MeOH extract of the leaf of the Indian plant *Phyllanthus niruri*, we isolated niruriside (**1**), a novel specific inhibitor of REV protein/RRE RNA. Three known compounds, phyllanthin, rutin, and kaempferol-3-*O*-rutinoside, were also isolated by solvent partition, column chromatography on Si gel and Sephadex LH-20 with various solvent systems, HPLC on YMC reversed-phase C_{18} column, and preparative TLC on Si gel under the guidance of the bioassay. The structure of niruriside (**1**) was established by spectroscopic methods as described below. The known compounds were identified by directly comparing their spectroscopic data with those of authentic samples and with published data.[16−18]



Niruriside (1)

Niruriside was isolated as a colorless powder. HRMS analysis revealed that it has a molecular formula of $C_{38}H_{42}O_{17}$, with a molecular weight of 770.2397. The UV absorption maximum at 280 nm was indicative of aromatic functionality with extended conjugation. The IR spectrum showed absorption bands at 3460, 1740, and 1720, which are characteristic of hydroxyl and ester groups. Four signals in its ^{13}C-NMR spectrum at δ 129.05, 129.00, 128.36, and 128.18, each of which represented two sp^2 tertiary carbons, suggested the presence of four pairs of equivalent CH groups. Together with two other sp^2 CH groups (δ_C 130.60, δ_H 7.38; δ_C 131.05, δ_H 7.38) and two sp^2 quaternary carbons (δ_C 133.79 and 134.13), they built two aromatic rings, which should be monosubstituted. The four carbonyl groups at δ_C 170.78, 170.68, 170.68, and 170.17 as well as four methyl groups (δ_C 20.83, 20.78, 20.62, and 20.58; δ_H 2.11, 2.11, 2.01, and 1.92) suggested the presence of four acetyl groups. The ^{13}C-NMR spectrum further revealed 12 carbon signals arising from a disaccharide moiety, whose anomeric carbon signals at δ 102.93 and 89.30 were characteristic of sucrose.[19] In addition, there were four vinyl CH groups (δ_C 147.29, δ_H 7.76; δ_C 145.96, δ_H

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Table 1. ^{13}C- and ^{1}H-NMR Data and Key C-H Long−Range Correlations of Niruriside (**1**) ($CDCl_3$, δ in ppm, J in Hz)

position	^{13}C	^{1}H	long-range C→H	long-range H→C
1A	64.37	4.20 (d, J = 11.8, H-A)		C-10‴
1B		4.08 (d, J = 11.8, H-B)		C-2, C-3, C-10‴
2	102.93		H-1B, H-1′	
3	79.05	5.33 (d, J = 7.8)	H-1B, H-5	C-4, C-9″
4	73.56	4.41 (t, J = 7.8)	H-5, H-3	C-3, C-5
5	80.92	4.21 (m)	H-4, H-6	C-4
6	63.85	4.50 (2H, d, J = 4.88)		C-4, C-5, C-9‴
1′	89.30	5.66 (d, J = 3.7)		C-1, C-5′
2′	72.65	4.78 (dd, J = 10, 3.7)	H-3′	C-3′, C-12‴
3′	69.82	4.01 (t, J = 9.8)	H-2′	C-2′, C-4′
4′	70.93	4.82 (t, J = 10)	H-3′	5′, C-6′, C-14‴
5′	68.48	4.22 (m)	H-1′, H-4	
6′A	62.55	4.14 (dd, J = 10.8, 2.3)	H-4′	C-16‴
6′B		4.09 (dd, J = 10.8, 5.6)		C-16‴
1″	134.13		H-3″, H-8″	
2″	128.18	7.56 (m)	H-7‴	
3″	129.00	7.38 (m)		C-1″, C-5″
4″	130.60	7.38 (m)	H-6″	
5″	129.05	7.38 (m)	H-3″	
6″	128.36	7.52 (m)		C-4″
7″	145.96	7.73 (d, J = 16.08)	H-8″	C-2″, C-8″
8″	117.15	6.48 (d, J = 16.08)	H-7″	C-1″, C-7″
9″	166.97		H-3	
1‴	133.79		H-5‴, H-8‴	
2‴	128.18	7.56 (m)		C-4‴, C-7‴
3‴	129.00	7.38 (m)	H-5‴	
4‴	131.05	7.38 (m)	H-2‴	
5‴	129.05	7.38 (m)		C-1‴, C-3‴
6‴	128.36	7.56 (m)	H-7‴	
7‴	147.29	7.76 (d, J = 16.05)	H-2‴	C-6‴, C-9‴
8‴	116.18	6.50 (d, J = 16.05)		C-1‴
9‴	167.13		H-6, H-7‴	
10‴	170.17		H-1A, H-1B, H-11‴	
11‴	20.58	1.92 (3H, s)		C-10‴
13‴	20.62	2.02 (3H, s)		C-12‴
14‴	170.68		H-4′, H-15‴	
15‴	20.77	2.11 (3H, s)		C-14‴
16‴	170.78		H-6′A, H-6′B, H-17‴	
17‴	20.83	2.11 (3H, s)		C-16‴

7.73; δ_C 117.15, δ_H 6.48; δ_C 116.18; δ_H 6.50) and two more ester carbonyls (δ_C 167.13 and 166.97).

On the basis of the above evidence and detailed NMR data (Table 1) studies including COSY, HETCOR, DEPT, HMBC, and COLOC, we proposed structure **1** for niruriside. The cinnamoyl groups were proven by the HMBC correlations (Table 1). The *trans* configurations between H-7″ and H-8″ and between H-7‴ and H-8‴ were confirmed by their $^{1}H-^{1}H$ coupling constants (16.1 Hz), respectively. Typical proton coupling constants and patterns of the sucrose moiety were observed in the ^{1}H-NMR spectrum, although the chemical shifts of most sugar protons and carbons were shifted downfield because of the esterification of the sugar hydroxyl groups. The doublet at δ 5.66, J = 3.7 Hz, which was assigned to the anomeric proton (H-1′) of glucosyl, indicated the α configuration of the glucosyl moiety. The NOE between H-3 at δ 5.33 and H-1A at δ 4.20 suggested the β configuration of the fructosyl moiety. Moreover, the connectivity between the two sugars was confirmed by the long-range coupling between C-2 (δ 102.93) of the fructosyl and H-1′ (δ 5.66) of the glucosyl moieties. The locations of the six acyl residues were mainly established by ^{13}C-^{1}H long-range correlations obtained from COLOC and HMBC experiments. Both cinnamoyl groups were attached to the fructosyl moiety, namely at C-3 and C-6, because of the long-range couplings between C-9″ (δ 166.97) and H-3 (δ 5.33) as well as between C-9‴ (δ 167.13) and H-6 (δ 4.50). One acetyl group was linked to C-1 of the fructosyl moiety due to the coupling of C-10‴ (δ 170.17) to H-1A (δ 4.20) and H-1B (δ 4.08). The other three acetyl groups were placed at C-2′, C-4′, and C-6′ of the glucosyl moiety, because we observed the couplings of C-12‴ (δ 170.68) to H-2′ (δ 4.77), C-14‴ (δ 170.68) to H-4′ (δ 4.82), and C-16‴ (δ 170.78) to H-6′A (δ 4.14) and H-6′B (δ 4.09). All of these attachments were well supported by significant downshifts of H-1A, H-1B, H-3, H-6, H-2′, H-4′, H-6′A, and H-6′B (0.5−1.5 ppm) compared with those of the corresponding signals of sucrose. Therefore, the structure of niruriside was determined to be β-D-(1-*O*-acetyl-3,6-*O*-*trans*-dicinnamoyl)fructofuranosyl α-D-(2,4,6-*O*-triacetyl)glucopyranoside.

Niruriside inhibited the binding of REV−protein to [^{33}P]-labeled RRE RNA with an IC_{50} value of 3.3 μM. To confirm its specificity for REV−RNA binding inhibitory activity, it was tested further for activity in an unrelated RNA−protein binding system, the R17 coat protein/operator RNA system, where the IC_{50} > 130 μM. Because niruriside demonstrated a high IC_{50}R17/IC_{50}-RRE ratio (>40), it was considered a specific REV/RRE binding inhibitor. However, niruriside did not protect CEM-SS cells at concentration levels up to 260 μM against acute HIV-1 infection using an XTT dye reduction assay.

Experimental Section

General Experimental Procedures. Solvents used for extraction, solvent partition, and column chromatography were ACS grade. CH_3CN for HPLC was

Fisher HPLC grade, and H_2O for HPLC was in-house de-ionized using a Barnstead Nanopure II system. TLC was performed on Kieselgel 60 F_{254} plates 0.2-mm thick (EM Science). For column chromatography, Si gel 60 (EM Science, particle size 40−63 μm) and Sephadex LH-20 (Pharmacia) were used. HPLC purification was performed on a C_{18} semipreparative column, S-7 μm, 250 × 20 mm i.d. (YMC Co.). The UV spectrum was taken on a Shimadzu UV2100 spectrophotometer; the IR spectrum was recorded on a Perkin-Elmer FT-IR 1800 spectrometer; Electrospray mass spectra (MS) were taken on a Finnigan TSQ7000 triple quadruple mass spectrometer, the high resolution FABMS analysis was performed with a Kratos MS50 mass spectrometer, and all ^{1}H- and ^{13}C-NMR spectra, including COSY, HETCOR, COLOC, and HMBC, were obtained on a Bruker AM-500 spectrometer (^{1}H, 500 MHz; ^{13}C, 125 MHz).

Plant Material. *P. niruri* L. was collected and identified by staff at the Bhogilal C. Shah Co., Indole, India. A voucher specimen is deposited at the herbarium of the company.

Extraction and Isolation. Dried and powdered leaves (100 g) of *P. niruri* were extracted by refluxing for 30 min successively with 100% MeOH, 80% MeOH, and 70% MeOH (1 L each). The pooled extracts were evaporated *in vacuo* to yield a dark, greenish residue (4 g). This solid extract was dissolved in 90% aqueous MeOH (222 mL) and then partitioned with hexane (3 × 222 mL). The aqueous MeOH layer was diluted with 44 mL of H_2O (75% MeOH) and partitioned against pre-equilibrated CCl_4 (3 × 266 mL). The CCl_4 extracts were combined and evaporated to give 210 mg of solid. The aqueous MeOH layer was further diluted with 42 mL of H_2O to a 65% MeOH solution and partitioned against pre-equilibrated $CHCl_3$ (3 × 208 mL). Both $CHCl_3$ and aqueous MeOH layers were pooled and evaporated to 400 mg and 2 g extracts, respectively.

The CCl_4 extract was subjected to column chromatography on Si gel, eluted with the CH_2Cl_2−MeOH−H_2O system (90:10:1−60:40:4). The fraction eluted with CH_2Cl_2−MeOH−H_2O (90:10:1) was concentrated and further chromatographed on a Sephadex LH-20 column ($CHCl_3$−MeOH, 1:1) to give a residue (75 mg), which was a mixture of two compounds according to TLC analysis. Separation of this mixture by preparative TLC using CH_2Cl_2−MeOH (95:5) afforded 16 mg of niruriside (R_f = 0.51) and 25 mg of phyllanthin (R_f = 0.82). The $CHCl_3$ extract was fractionated by column chromatography on a Sephadex LH-20 ($CHCl_3$−MeOH, 1:1). One of the obtained fractions (25 mg) appeared to contain mainly niruriside, based on TLC and HPLC analysis. This fraction was further purified by column chromatography on a MCI-gel (80% MeOH) to give 12 mg of pure niruriside. The aqueous MeOH extract (600 mg) was chromatographed repeatedly on a Sephadex LH-20 column and eluted with CH_3CN−H_2O (4:1) to give a very hydrophilic residue (300 mg). Separation of this residue on a YMC semipreparative HPLC column eluted with CH_3CN−H_2O (2:3) gave 33 mg of rutin (t_R = 16.5 min) and 10 mg of kaempferol 3-*O*-rutinoside (t_R = 25.5 min).

Niruriside (1): white amorphous powder; IR (KBr) ν max 3460, 2930, 1746, 1720, 1636, 1578, 1498, 1370, 1234, 1164, 1036, 768 cm^{-1}; UV λ max (log ϵ) (MeOH) 204 (3.47), 217 (3.44), 221 (sh 4.38), 280 (4.53) nm; HRFABMS found (*m*/*z*) 770.2397 [M]$^+$, calcd for $C_{38}H_{42}O_{17}$, 770.2421; ^{1}H and ^{13}C NMR, see Table 1.

REV/RRE Binding Assay. REV protein was expressed in *Escherichia coli* and prepared essentially as described.[20] The HIV-1 RRE region was cloned between the T7 and T3 promoters in the Bluescript KS+ plasmid (Stratagene). In vitro transcribed, [^{33}P]-radiolabeled RRE RNA was incubated with potential inhibitors and REV protein. Uninhibited RRE RNA forms a complex with REV protein that is then bound to nitrocellulose filters and is quantitated by liquid scintillation counting as described.[4,5,21] This assay was automated and adapted for high-throughput screening of synthetic compounds and natural product extracts. Niruriside was identified in this screen as an inhibitor of REV/RRE binding.

R17 Coat/Operator RNA Assay. The specificity of the REV/RRE-binding inhibitory activity was confirmed by the R17 coat/R17 hairpin RNA binding assay. Purification of R17 coat protein was performed as described.[22] Synthesis of R17 short hairpin genomic RNA and assay of R17 coat/RNA binding were carried out as reported.[23] [^{33}P]-Radiolabeled R17 operator RNA binding to R17 coat protein was measured using nitrocellulose filters as described for the [^{33}P] RRE RNA/REV protein system.

HIV Assay. The anti-HIV activity of the REV/RRE binding inhibitors was evaluated according to Weislow *et al.*[24] Briefly, CEM-SS cells were infected with HIV-1 RF and incubated at 37 °C in the presence of serial dilutions of compounds. Six days later, XTT and *N*-methyl-phenazonium methosulfate were added to each well, and the plates were incubated for 4 h to allow for XTT formazan production. Cell viability was quantified by light absorbance at 450 nm using a reference wavelength of 650 nm. Data were expressed as a percentage of formazan produced in test wells compared to formazan produced in wells of untreated control cells. EC_{50} values were calculated as the concentration of compound that increased the percentage of formazan production in virus-induced cells to 50% of that produced by uninfected cells. Likewise, the cytotoxicity was calculated as the concentration of compound that decreased the percentage of formazan produced in uninfected cells to 50% of that produced in untreated cells.

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