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Isolation of anti-HIV-1 lignans from *Larrea tridentata* by counter-current chromatography

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Abstract

Several lignans, mostly new, were isolated from *Larrea tridentata* by assay-guided counter-current chromatography (CCC). Using the secreted alkaline phosphatase bioassay of HIV Tat transactivation and the two-phase hexane–ethyl acetate–methanol–water solvent system, two major components (*Gr* and *Lo*) were identified as anti-HIV active principles. The chemical structures of the constituents of *Gr* (G_1–G_4) and *Lo* (L_1–L_4) were determined by GC–MS and NMR. After optimization of isolation conditions, a large-scale isolation with the chloroform–methanol–water system yielded five constituents (FB_1–FB_5). The most predominant anti-HIV compound FB_2 (denoted *Malachi* 4:5–6 or mal.4), which occurs in 0.23% yield, was separated from its FB_1 isomer (0.13% yield). Compound FB_4 and two tricyclic lignans (FB_3 and FB_5) were also isolated in a substantial amount for further testing of their anti-HIV activities. These compounds may represent a new class of anti-HIV agents with important clinical relevance.

Keywords: Human immunodeficiency virus; *Larrea tridentata*; Secreted alkaline phosphatase assay; Lignans; 3′-O-Methylnordihydroguaiaretic acid

1. Introduction

The desert plant *Larrea tridentata* (DC.) Cov. (synonym: *Covillea tridentata* or *L. mexicana* Moric.) or creosote bush (Zygophyllaceae) is a perennial shrub widely used in traditional medicine among American indians for various ailments including digestive disorders, rheumatism, venereal diseases, and sores [1]. The earliest report of occurrence of a lignan, nordihydroguaiaretic acid (NDGA), in the creosote bush dates back to 1945 [2]. Since then, *Larrea* species have been extensively investigated [3]. A number of lignans and other compounds have been identified in these species [4], most notably *L. divaricata*, which yields NDGA, heminordihydroguaiaretic acid (HNDGA) and norisoguaiacin [5], with undetermined activity.

Lignans exhibit a wide range of biological properties including antifungal, antimicrobial and antitumorigenic activities, and inhibition of many enzyme systems [6]. Most interestingly, some of

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these lignans have been associated with remarkable antiviral activities [7]. These activities have been recently reviewed [7]. *L. tridentata* is a source for several lignans including nordihydroguaiaretic acid (NDGA), a useful antioxidant for food and pharmaceuticals [8,9]. Recently, NDGA and several other non-sulfhydryl antioxidants were shown to inhibit cytokine-stimulated promoter activity of HIV transcriptional processes [10]. Despite these reports, the anti-HIV activities of *L. tridentata* and several congeners of NDGA previously isolated from this plant [4] have not been thoroughly investigated. These studies require pure preparation of plant lignans as well as suitable assay systems for examining the anti-HIV activity.

HIV Tat is a potent transcription activator encoded by the human immunodeficiency virus-1 (HIV-1) and is required for the replication of the deadly virus [11]. Tat-regulated transactivation has since become an attractive target for development of anti-HIV drugs [12]. A powerful bioassay, involving constructs of HIV LTR promotor and reporter gene, the secreted alkaline phosphatase (Seap) and CMV promotor-driven Tat, was established [13] for screening potential HIV inhibitors. By using a highly efficient counter-current chromatography (CCC) and Seap bioassay as purification guide, we have isolated a dozen lignans from the active fractions of *L. tridentata*. The biological activity of one of these compounds, mal.4, has been established. Mal.4 was found to be a strong inhibitor of HIV transcription, HIV Tat-regulated transactivation and HIV replication [14]. These lignans, in general, by their specific inhibition of proviral transcription may represent a new class of potent anti-HIV drugs or serve as a chemotype for drug development.

2. Experimental

2.1. Cell line and plasmids

COS-7 cells with SV40 origin of replication were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal calf serum (FCS) and antibiotic. Cells were incubated in a humidified 95% O_2–5% CO_2 incubator at 37°C. The plasmid pBC12/HIV/SEAP containing the Tat-sensitive HIV-LTR promoter with Seap reporter gene but no Tat-coded function was used to express Seap basal activity; pBC12/CMV/t2 supplied the Tat-coded function (CMV/Tat), i.e., the induced Seap level; pBC12/RSV/SEAP containing the constitutive Tat-insensitive LTR promoter of Rous Sarcoma Virus (RSV) served as a positive control. All plasmids were a generous gift form Dr. Bryan Cullen (Duke Medical Center).

2.2. Chemical reagents

Diethanolamine (Cat. No. 31589) *p*-nitrophenyl phosphate (Cat. No. 71768) were purchased from Fluka BioChemika, and L-homoarginine (Cat. No. H-1007) was from Sigma. The lipospermine DOGS (Transfectam, Cat. No. E123 A, Promega) was utilized for DNA transfection.

2.3. Plant materials and preparation of testing materials

The leaves of *L. tridentata* were collected in summer in Arizona and sun-dried for several weeks. The plant materials were ground in a 3-mm screen Wiley mill and the powder was weighed and immediately used for extraction. All plant crude extracts and fractions generated by fractionation were dried, weighed, and monitored by SiO_2 thin-layer chromatography (TLC) with cerium sulfate charring [2% $CeSO_4$ (w/v) in 5.6% H_2SO_4 (v/v)]. The test plant materials were prepared as follows: The total plant extract (sample of residues from chloroform–methanol extraction) was defatted with hexane. The hexane-insoluble material (HI) and the samples from CCC fractionation dried to residues were dissolved in a 10% dimethylsulfoxide (DMSO) solution made in calcium/magnesium-free phosphate buffer saline (PBS) prior to testing with Seap assay. The suspension was centrifuged and the stock solution was filter-sterilized using a Millex-GS 22-μm filter (Millipore). Appropriate

dilutions of the stock solution were prepared in a final DMSO concentration of 0.2% in PBS to obtain the various concentrations of the test materials.

2.4. The secreted alkaline phosphatase (Seap) assay

The principle of the Seap assay is illustrated in Fig. 1. The screening of plant materials for inhibition of Tat transactivation was achieved with the Seap assay originally described by Berger et al. [13]. Briefly, triplicate samples of COS cells were co-transfected with a mixture of the plasmid DNAs, pBC12/HIV/SEAP and pBC12/CMV/t2 (coding for Tat function) in a 2:1 ratio, using the newly introduced lipospermine procedure [15]. Cells were incubated for 12–15 h after transfection. Test materials were added to the transfected cells at the appropriate concentrations in a final DMSO concentration of 0.2%, and samples were incubated for 48 h. Seap was analyzed on a 250-μl aliquot removed from COS cell culture supernatants as previously described [13]. The percent inhibition of Seap expression was calculated at time $t = 30$ min of Seap activity as follows:

$$\%\,\text{Inhibition} = 100 - [(CT^{+} - C^{+})/(CT^{-} - C^{-})] \times 100$$

where C^{-} is control sample (no DNA, no drug), CT^{-} control sample (+DNA, no drug), C^{+} drug-treated sample (no DNA, +drug), and CT^{+} drug-treated sample (+DNA, +drug).

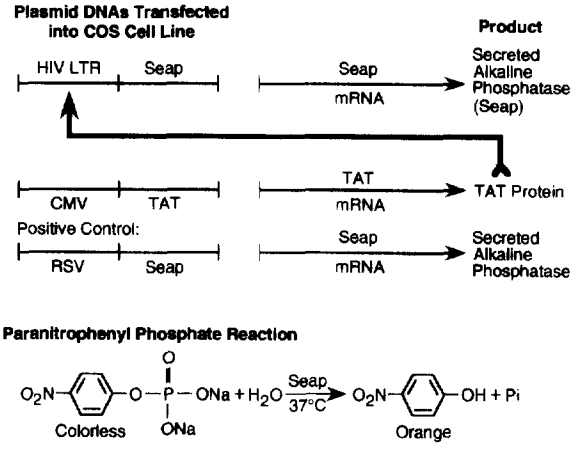

Fig. 1. Principle of Seap assay. COS cells with SV40 origin of replication are co-transfected with the vectors pBC12/HIV/SEAP containing the Tat-sensitive HIV-LTR promoter, and pBC12/CMV/t2 supplying the Tat-coded function, in 2:1 ratio. Under the control of the cytomegalovirus (CMV) promoter, the Tat gene is transcribed into mRNA and translated into Tat protein which binds to HIV-LTR promoter. This binding induces the transcription and expression of Seap enzyme, which is excreted into the culture medium. The pBC12/RSV/SEAP vector containing the constitutive Tat-insensitive LTR promoter of Rous Sarcoma Virus (RSV) serves as a positive control. Seap enzyme is quantitated by a colorimetric reaction in the presence of *para*-nitrophenyl phosphate substrate. Tat-induced expression of the Seap enzyme is a measure of the transactivation of Tat protein [12]. A specific inhibitor of Tat transactivation is expected to decrease Seap levels by interfering with the usage of HIV-LTR template in Tat-regulated transactivation without affecting the Tat-insensitive RSV promoter.

2.5. Instrumentation and columns of counter-current chromatography

Three apparatuses were used in the present studies. The first involved the versatile cross-axis coil planet centrifuge (CPC) previously described [16]. Both coils had 570 ml total capacity (285 ml each). The second apparatus was the high-speed CCC centrifuge with an Ito multilayer coil (P.C. Inc., Potomac, MD, USA) previously described [17]. The column was 1.6 mm I.D. and 331 ml capacity with β value ranging from 0.5 at the internal terminal to 0.85 at the external terminal (β is a parameter describing the partition efficiency and retention of the stationary phase). This column was utilized for the semi-preparative separations. Finally, the triplet CPC manufactured at the NIH and described in detail elsewhere [18] was utilized to achieve the final purification of the components, due to its high resolution and effectiveness.

The solvent was pumped with a Milton Roy metering pump (Model 196-31) (LTC/Milton Roy, FL, USA). The eluent was monitored at 254 nm with a UV detector (Model Uvicord S, LKB Instruments, Stockholm, Sweden) with a strip chart recorder (LKB Instruments).

3. Results and discussion

3.1. Induction of Tat-regulated Seap expression

A standard Seap assay in the absence of drug was initially run to assess the efficiency of transfection. The results of these studies (Fig. 2) indicate a nearly 65-fold increase in Tat-induced Seap expression after 1 h compared to the control (no DNA) levels of the enzyme at the baseline, which were similar to those of the nonselected gene (HIV/Seap) alone. The positive control, RSV/Seap, containing the Tat-insensitive LTR promoter, showed a similar time course increase in Seap expression with a maximum of 40 milli-units/well after 1 h.

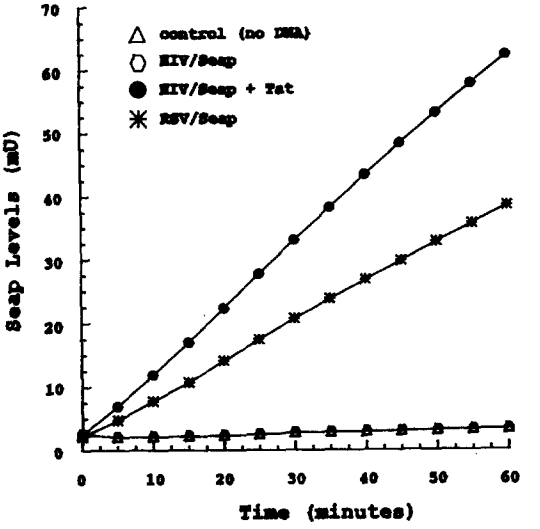

Fig. 2. Induction of secreted alkaline phosphatase (Seap) expression in Seap standard assay. COS cells were maintained in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal calf serum (FCS) and antibiotic. Triplicate cell samples were seeded at a density of ca. 1.5×10^5 cells per well in Linbro 24 flat-bottom wells of 17 mm diameter and incubated in a humidified 95% O_2–5% CO_2 incubator at 37°C until they reached 50% confluency. Subconfluent cells were transfected using the lipospermine procedure [15]. Briefly, the medium of the subconfluent cells was aspirated and replaced by 300 μl of fresh complete medium (IMDM supplemented with 10% FCS). COS cells were transfected with either pBC12/HIV/SEAP alone (0.4 μg/well) or pBC12/CMV/t2 (coding for Tat function) at 0.2 μg/well + pBC12/HIV/SEAP (0.4 μg/well) or with buffer alone (control samples with no DNA). Cells and plasmid DNAs were kept in contact for 3–4 h, after which 700 μl of fresh complete medium was added. Cells were then incubated for 48 h. At the end of this incubation, a 250-μl aliquot was removed from COS cell culture supernatants and Seap was analyzed as described elsewhere [13]. Briefly, samples of culture supernatants were heated at 65°C for 5 min to selectively inactivate endogenous phosphatases (Seap is heat stable), and centrifuged in a microfuge for 2 min. An amount of 100 μl of 2X Seap assay buffer (1.0 *M* diethanolamine, pH 9.8; 0.5 m*M* $MgCl_2$; 10 m*M* L-homoarginine) was added to a 100-μl aliquot sample and mixed by vortexing. The solution was transferred into a 96-well flat-bottom culture dish (Corning). Then, 20 μl of pre-warmed substrate solution (120 m*M* *p*-nitrophenyl phosphate dissolved in 1X Seap assay buffer) was dispensed with a multipipetter into each well containing the reaction mixture. The absorbance at 405 nm (A_{405}) of the reaction was read at 5-min intervals at 37°C over the course of 60 min on an EL340i microplate reader (Bio-tek Instruments) with 5-s automatic shaking before each reading. The change in absorbance was converted into milli-units (mU) of Seap expression [13] and plotted against time.

3.2. Assay-guided fractionation of the first batch of plant materials, a pilot study

In a pilot study, the fractionation of plant materials (101.4 g) started with successive macerations of the powder using a mixture of chloroform–methanol. This total extract was tested with Seap assay and displayed a dose-dependent inhibitory activity of Tat transactivation with an $IC_{50} \approx 600$ μg/ml (the IC_{50} is the concentration exhibiting 50% inhibitory activity, and is a measure of drug potency; see Figs. 3 and 4). The same extract was also found to inhibit HIV replication and protect the human lymphoblast CEM-SS cells against HIV-induced cell death (data not shown) in the formazan assay [14,19]. However, this extract exhibited a narrow range of activity and a marked intrinsic cytotoxicity. Based on these test results, the total extract was defatted with hexane to remove fats, waxes, and volatile constituents potentially toxic to culture cells. The hexane-insoluble (HI) fraction was tested with Seap assay. The results, illustrated in Fig. 4, show a concentration-dependent activity with an $IC_{50} = 250$ μg/ml for this fraction.

A working sample of 10 g HI residue was partitioned in chloroform–water and generated three fractions: (1) an inactive aqueous phase residue; (2) an enriched chloroform phase; and (3) an insoluble residue. The latter residue was further treated with ethyl acetate (EtOAc), and

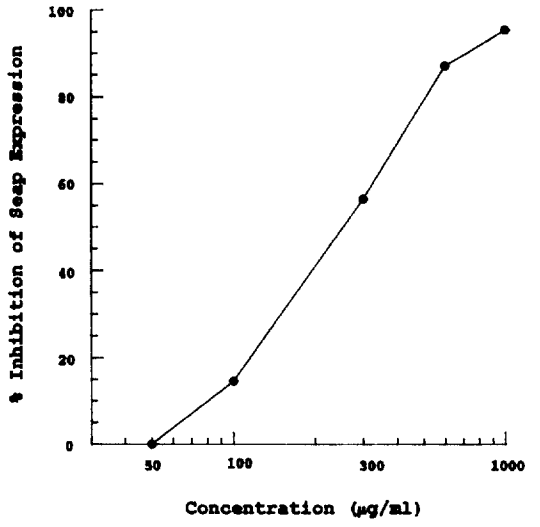

Fig. 3. Inhibition of Tat transactivation by crude extracts of creosote bush in Seap assay. Triplicate samples of COS cells were transfected as described in Fig. 2 with a mixture of pBC12/HIV/SEAP and pBC12/CMV/t2 (coding for Tat function) in 2:1 ratio, using the lipospermine procedure [15]. Cells were incubated for 10–12 h after transfection before the test material was added at various concentrations. The test plant material [hexane-insoluble (HI) residue] was prepared as described in the Experimental section. The samples were incubated for 48 h, after which the Seap assay was performed on a 250-μl aliquot of COS cell culture supernatants. The percent inhibition of Seap expression was calculated at time $t = 30$ min as described in the Experimental section. Each point represents the average of nine determinations ($n = 3$).

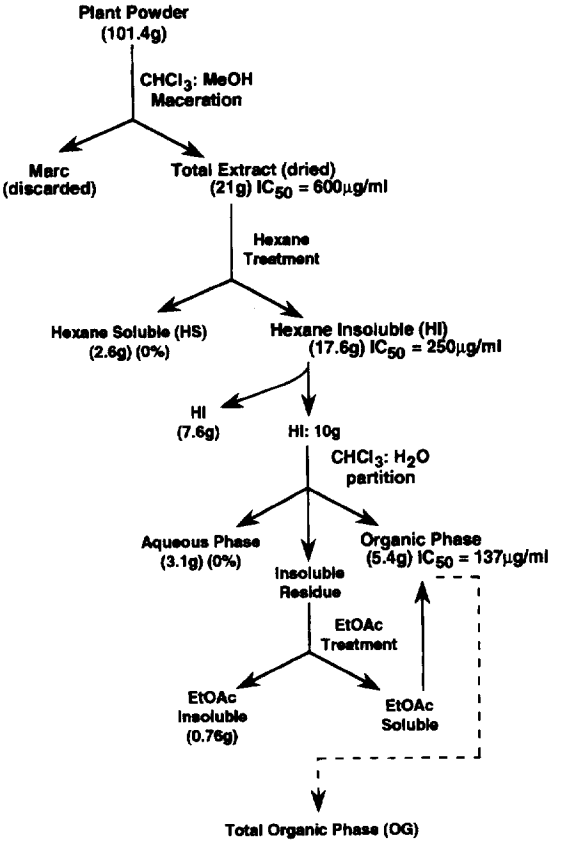

Fig. 4. Assay-guided fractionation of the creosote bush in a pilot study. Plant extracts were tested stepwise for inhibition of HIV-Tat transactivation using the secreted alkaline phosphatase assay as described in the Experimental section.

yielded 0.76 g of EtOAc-insoluble materials and an EtOAc-soluble fraction. Based on the similarity of the TLC patterns, the EtOAc-soluble fraction was combined with the chloroform fraction, yielding 5.4 g of an active organic phase (OG) with $IC_{50} = 137$ μg/ml. Thus, chloroform–water partition of the hexane-insoluble fraction generated an enriched organic phase with a four-fold increase in activity compared with the original total extract.

3.3. *Isolation of the anti-HIV components (Gr* and *Lo)* by counter-current chromatography

Further fractionation of the major components from the organic phase (OG) was achieved by counter-current chromatography (CCC) using the versatile cross-axis coil planet centrifuge (CPC) with a pair of multilayer coils mounted at the off-center position [16]. The solvent system was a mixture of hexane–EtOAc–methanol–0.5% NaCl in the 6:4:5:5 ratio (Fig. 5), with the organic layer as the mobile phase. This solvent system was denoted hexane system. The ratio of this system was determined based on the values of the partition coefficient (K). Here, K was defined as the ratio of the activity of the component(s) in the aqueous phase over the activity of the same component(s) in the organic phase. In the initial pilot studies, K values were primarily assessed by Seap bioassay on a trial-and-error basis since the active components had not been identified. The above hexane system ratio al-

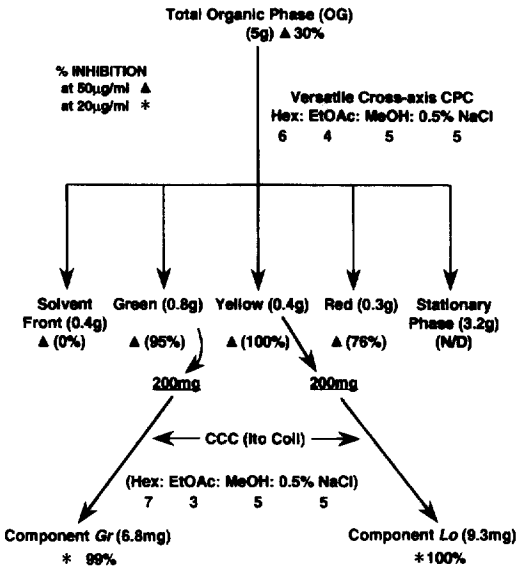

Fig. 5. Assay-guided CCC fractionation of the organic phase (OG) fraction. The CCC fractionation of the OG fraction generated two major active components (*Gr* and *Lo*) from fractions Green and Yellow, respectively, as described in the text. All intermediate CCC fractions and the components of the end-product were tested in Seap assay at concentrations of 50 μg/ml (▲) and 20 μg/ml (*), respectively.

lowed a K value of 0.7, which was within the suitable range of a good CCC separation [20].

Five grams of the OG fraction from the pilot studies was fractionated by CCC with the hexane system, with approximately 68% retention. The fractions were monitored by SiO_2 TLC using the analytical chloroform system, chloroform–methanol–water (lower phase) in 80:20:10 ratio, and pooled into five batches based on the similarity of TLC patterns. These batches included: solvent front, fraction green, fraction yellow, fraction red and stationary phase (SP). All these fractions were further Seap-assayed at 50 μg/ml for inhibition of HIV Tat transactivation. The percent inhibition is given in parentheses in Fig. 5.

Further analysis of fractions green and yellow (95 and 100% activity, respectively) led to the identification of two major components of different R_F values in the analytical chloroform system, i.e., chloroform–methanol–water (lower phase) in 80:20:10 ratio: (1) an $R_F = 0.8$ component generated from fraction green, and (2) an $R_F = 0.6$ component from fraction yellow. These components were separately fractionated by CCC with the hexane system in 7:3:5:5 ratio using the highly efficient type-J coil planet centrifuge known as the Ito multilayer coil separator–extractor [17]. This solvent ratio gave a K value of approximately 1.5, which was determined by TLC analysis as the ratio of the concentration of the component of interest in the upper organic phase over the concentration of the same component in lower aqueous phase. From the initial 200 mg of fraction green, only 6.8 mg of clean $R_F = 0.8$ component was recovered with ca. 80% purity. This constituted 0.05% yield based on the original weight of plant powder. The $R_F = 0.8$ component was termed component *Gr*. Under similar conditions, 200 mg fraction yellow generated a widespread $R_F = 0.6$ component (Fig. 6) whose elution peak tail (fraction nos. 125–131) afforded 9.3 mg of a fairly clean mixture, denoted component *Lo*.

3.4. Inhibition of HIV cytopathic effects by components Gr and Lo

Components *Gr* and *Lo* and their respective parent fractions (green and yellow) were subjected to the NCI soluble-formazan assay [19] for direct antiviral activity in HIV-challenged CEM-SS human lymphoblast cell line. All test materials exhibited a concentration-dependent inhibitory activity of HIV cytopathic effects. Table 1 illustrates duplicate data of the peak of activity of these fractions. Component *Gr*, at a concentration of 0.75 μg/ml, caused 58% protection (cell viability) against HIV as opposed to 15.5% viability in drug-free infected cells. Interestingly, the parent fraction green, at a concentration four times less, exhibited greater activity (66.6%) than component *Gr*. This suggests the presence of unresolved anti-HIV constituents in the fraction green, which may act synergistically with component *Gr* in protecting CEM-SS cells from HIV infection. However, the crude green fraction had expectedly a higher intrinsic cytotoxicity around 40%. The intrinsic cytotoxicity is defined

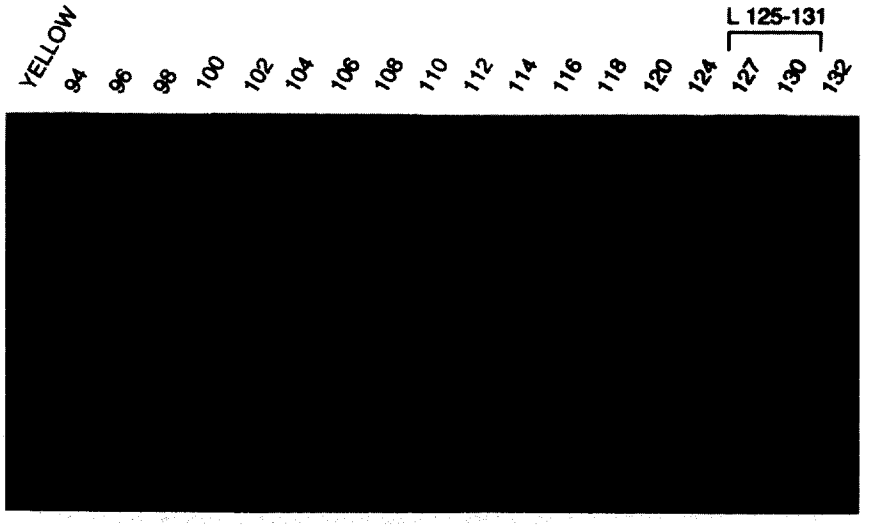

Fig. 6. Silica gel TLC analysis of component *Lo* from fraction yellow. CCC fractions of component *Lo* were analyzed by TLC. The TLC plates (Whatman 250-μm layer backing aluminum UV_{254} plate, Cat. No. 4420222) were developed in the chloroform analytical system consisting of a mixture of chloroform–methanol–water (lower phase) in 80:20:10 ratio. The plates were visualized by cerium sulfate charring [2% $CeSO_4$ (w/v) in 5.6% H_2SO_4 (v/v)]. The R_F value of component *Lo* was 0.6 in this system.

here as: 100% − % viability of uninfected cells in the presence of the drug.

The effects of the component *Lo* were more interesting. At a concentration as low as 0.187 μg/ml, this component exhibited a stronger inhibitory activity than *Gr* at 0.75 μg/ml. The viability was nearly 87% on average when cells were challenged with HIV in the presence of component *Lo* while the viability for drug-free cells infected with HIV was 14%. Furthermore, this component displayed the least intrinsic compound cytotoxicity (ca. 9%) in uninfected CEM-SS cells.

3.5. Structural analysis of several compounds originating from components Gr and Lo and anti-HIV activity of mal.4

The constituents of components *Gr* and *Lo* were identified by GC–MS and NMR analysis [21]. These studies revealed a mixture of four related lignans (G_1–G_4) and several fatty acids for component *Gr* (Fig. 7). These lignans are derivatives of nordihydroguaiaretic acid (NDGA), with varying O-methyl and O-acetyl substitution. Two tricyclic lignans, 3′-demethoxyisoguaiacin and norisoguaiacin, and ayanin were also identified in component *Gr*. Despite its remarkable anti-HIV activity, further purification of fraction green was found to be more difficult, due to the high complexity of this mixture. Optimization of the isolation conditions is underway to resolve the active constituents of this fraction.

Much effort was devoted to the purification of the more polar component *Lo* from fraction yellow. The structural analysis of this component revealed a mixture of four lignans, L_1–L_4 (Fig. 7), which are structurally related to the constituents of component *Gr*, but with fewer O-methyl substitutions. The 3′-O-methyl substituted lignan, denoted "Malachi 4:5–6" (mal.4), was 76% (7 mg) of the mixture [21], which represents approximately 0.02% yield based on the starting weight of plant powder.

Mal.4 was selected for a full range of anti-HIV screening. The results described elsewhere [14] indicate that: (1) mal.4 suppresses HIV cytopathic effects and protects normal lymphoblastoid cells (CEM-SS cells) against HIV-induced

Table 1
Inhibition of HIV-1 cytopathic effects by crude fractions of *L. tridentata* using the soluble formazan assay[a]

Test compound	Concentration of the test compound which yielded maximal protection against HIV (μg/ml)	Percent of live cells at day 6		
		Uninfected cells plus test compound	HIV-infected cells plus test compound	HIV-infected cells with no test compound
Fraction green	0.187	58.6	66.6	16.8
Component Gr	0.75	79.9	67.2	16.8
Component Gr	0.75	70.3	48.6	14.1
Fraction Yellow	0.187	60.1	57.4	14.1
Component Lo	0.187	91.8	86.1	14.4
Component Lo	0.187	89.7	87.1	14.4

[a] The formazan assay has been previously described [19]. Briefly, human lymphoblast CEM-SS cells are challenged with HIV in the presence of the test drug, and incubated for one week. If the drug inhibits HIV production, CEM-SS cells are protected from virus-induced cell death. The tetrazolium (XTT) reagent is metabolically reduced by the viable cells to yield a colored formazan product which is measurable by colorimetry at 450 nm. In this experiement, triplicate samples of CEM-SS cells (5000) were plated in a 96-well microtiter plate. Appropriate concentrations of test compounds were added in a final volume of 100 μl calcium/magnesium-free PBS in 5% DMSO. Control samples received PBS alone. Five minutes later, 500 H9 cells infected with HIV-1 or normal H9 cells were added to the wells containing the appropriate drug concentrations. The microtiter plates were incubated at 37°C in 95% O_2–5% CO_2 for 6 days, after which a 50-μl mixture of XTT and N-methylphenazonium methosulfate (PMS) was added. The plates were incubated for an additional 4-h period for color development (XTT formazan production). The plates were sealed, their contents were mixed by automatic shaking and the A_{450} of samples was determined on a microplate reader. Each value represents the average of three determinations. No statistically significant difference was found between the means of the duplicate values of the uninfected cells and HIV-challenged cells, in the presence of test compounds. In contrast, there was a significant difference ($p < 0.05$) between HIV-infected samples in the presence or absence of test compounds.

cell death; (2) the compound inhibits HIV replication in all five HIV strains examined, including drug-resistant strains, and primary isolates from patients with acute AIDS. One of the molecular mechanisms of action of mal.4 has been elucidated. The compound interferes with the binding of the Sp1 protein to Sp1 sites in HIV-LTR promoter, which leads to a blockade of the HIV transcription machinery, and thus, the suppression of HIV replication [14].

3.6. Optimization of CCC conditions for large-scale isolation of several plant lignans

The low recovery of both components *Gr* and *Lo* and mal.4 prompted the need for an optimization of the purification conditions. This involved changes in the nature and the ratio of the solvent system, and changes in the physical parameters of the CCC coil. The extent of spread of the *Lo* component assessed by TLC (Fig. 6) revealed that the hexane system had reached a limit in resolving this component. From this TLC analysis, several conclusion were drawn: (1) the horizontal spreading of $R_F = 0.6$ component (x-axis) direction in numerous fractions having the same R_F confirmed the early finding that a family of several chemically related compounds was involved; (2) these compounds could be partially resolved by CCC with the hexane system, but not by TLC when developed in the analytical chloroform system ($CHCl_3$–methanol–water, 80:20:10-lower phase); (3) however, due to its remarkable resolution capability of the $R_F = 0.6$ component in the Y-axis direction, this analytical chloroform system was considered as a suitable system for future CCC runs. Based on these observations, it became apparent that a more efficient separation would require a judicious combination of hexane and chloroform solvent systems.

A large-scale isolation of the active con-

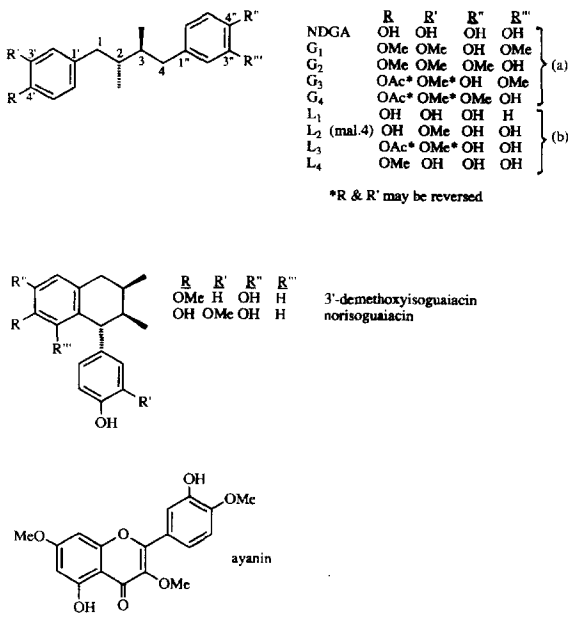

Fig. 7. Structures of some plant lignans isolated from *L. tridentata*. (a) Compounds G_1–G_4 from component *Gr*; (b) compounds L_1–L_4 from component *Lo*. The structures of other compounds from component *Gr* (norisoguaiacin, 3'-demethoxyisoguaiacin) and the flavone ayanin are also shown.

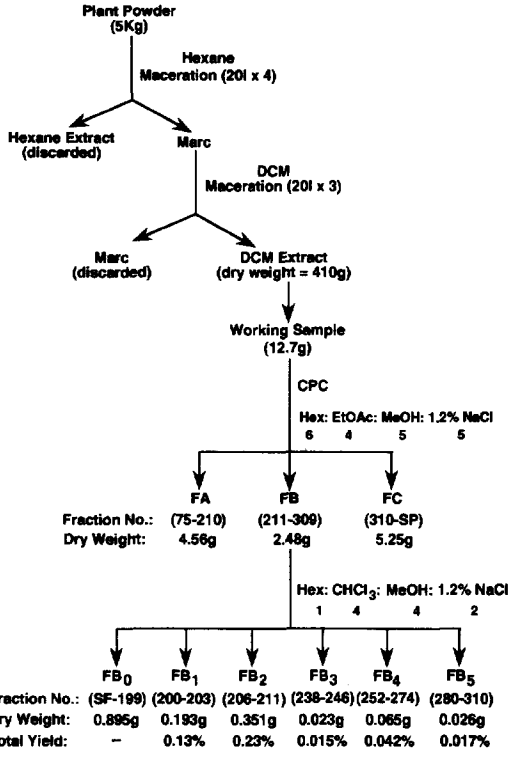

Fig. 8. Large-scale isolation of the active constituents of *L. tridentata* after optimization of isolation conditions. A working sample of 12.7 g of the DCM extract was further fractionated by CCC on the large CPC model using the hexane system to generate fraction FB (2.48 g) enriched with $R_F = 0.6$ major component. The various constituents of this component were resolved with the chloroform system as described in the text. This generated compounds FB_1–FB_5 in high purity.

stituents of the creosote bush was undertaken to verify experimentally these observations. Five kg of plant powder was treated with 20 l hexane three times. The hexane-soluble fraction was discarded (Fig. 8) and the dry marc was extracted three times by successive macerations with 20 l of dichloromethane (DCM). This afforded 410 g of DCM total extract. A working sample of 12.7 g of this residue was initially fractionated by CCC on the large-capacity versatile cross-axis CPC [16] using the hexane system in 6:4:5:5 ratio with 1.2% NaCl in the stationary aqueous layer. The presence of salt in the aqueous phase significantly lowered the propensity for emulsification and improved the retention (70%) of the stationary phase. Using the upper phase (organic layer) as the mobile phase, three major fractions were identified (Fig. 8) based on the elution order and TLC patterns. These fractions included: (1) FA (4.56 g), consisting of the early and most hydrophobic components, and component *Gr*; (2) FB (2.48 g), enriched with component *Lo* of interest; and finally, (3) FC (5.25 g), the column content, consisting of most polar components.

Further purification of fraction FB was achieved by CPC using the chloroform system (hexane–chloroform–methanol–1.2% NaCl) in 1:4:4:2 ratio, with the organic layer as a mobile phase. The sample was saturated with salt before injection. The presence of hexane in the chloroform system was required to account for the hydrophobicity factor introduced by the salt, and to bring the partition coefficient into the 1–2 operating range. With 45% retention, this system permitted a remarkable resolution of most con-

stituents (FB_1 to FB_5) of fraction FB, as illustrated in Fig. 9. The structure of these compounds was determined by both GC–MS and NMR. This study revealed that isomers FB_1 (0.193 g) and FB_2 (0.351 g) were identical to compounds L_4 (4′-O-methyl-NDGA) and L_2 (3′-O-methyl-NDGA, mal.4), respectively [21], from the early pilot studies (Fig. 7). These two lignans occurred in 0.13% (FB_1) and 0.23% (FB_2) yield, based on the starting weight of plant powder. The initial pilot studies had afforded only 0.02% yield of mal.4. This sharply contrasts with the current 0.23% yield obtained after improvement of the isolation conditions, which represents a nearly twelve-fold enrichment of mal.4. The relatively polar tricyclic lignans FB_3 and FB_5 (Fig. 9) purified from this CCC run occur in 0.015 and 0.017% yield, respectively. FB_5 has been described previously [22], in contrast to FB_4, which is structurally identical to L_1 (Fig. 7) and appears to be a new lignan. This compound occurs in relatively higher yield (0.042%) and has been crystallized [21]. The yellow flavone ayanin (Fig. 7), previously described in other plants [23,24] but apparently not in *Larrea* species, was also isolated in these studies, and occurs in ca. 0.007% yield (chromatogram not shown). This compound was devoid of activity in our screening assay.

Fig. 10 lists the chemical structures of all 16 constituents identified or isolated from this plant in the present studies, with their various denotations and an indication of the new structures. Further studies to resolve the active constituents of the more lipophilic component *Gr* and the investigation of the anti-HIV activity of the

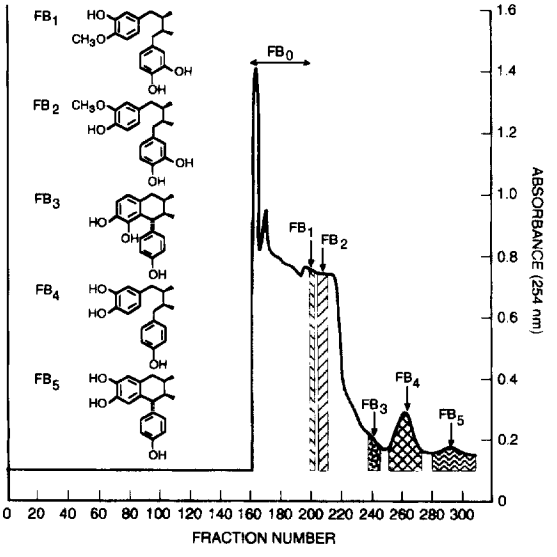

Fig. 9. Chromatogram of the large-scale isolation and structure of compounds FB_1–FB_5. The CCC fractions containing the two isomers FB_1 and FB_2 were analyzed by silica gel TLC with the chloroform analytical system as follows: an aliquot from each fraction was diluted with methanol and adjusted to a value of 2.22 OD at A_{280} in a Beckman spectrophotometer (Model 2400). A 5-μl aliquot of this solution was spotted on a TLC plate and analyzed as described in Fig. 6. The R_F values for FB_1 and FB_2 were 0.57 and 0.55, respectively. Structures of the compounds were determined by GC–MS and NMR [21].

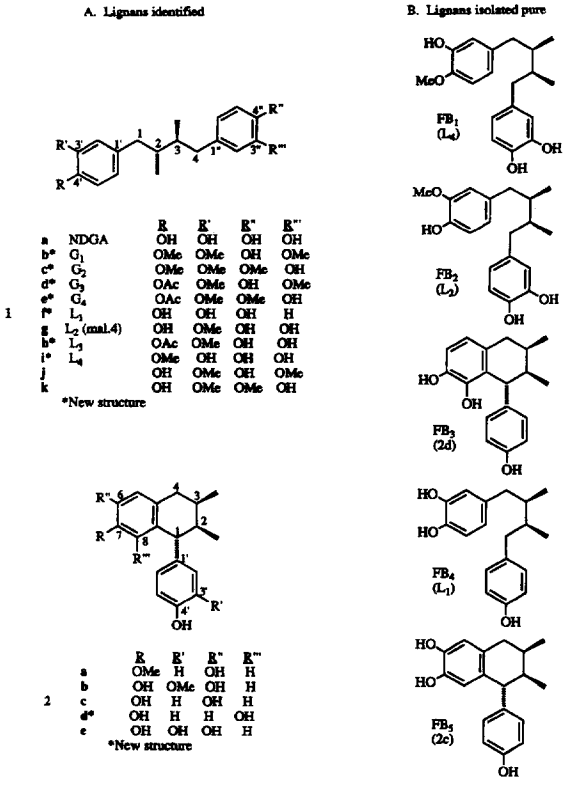

Fig. 10. Chemical structures of 16 lignans identified or isolated from *L. tridentata*. Compounds **1j**, **1k** were recently identified from fraction FA and compound **2e** from fraction FC. Both fractions result from the large-scale fractionation of 12.7 g hexane-treated plant material as illustrated in Fig. 8.

tricyclic lignans are in progress and will be reported separately.

Conclusion

The results of the present studies indicate that the desert creosote bush, *L. tridentata*, is a source for new lignans with anti-HIV activity. These compounds appear to be the first plant-derived agents to inhibit Tat-induced transactivation. The screening Seap bioassay, which guided the isolation of these compounds, was selected based on the phytopharmacological criteria of simplicity, sensitivity, reproducibility, and selectivity [25]. This specific assay, coupled with the highly efficient counter-current chromatographic method, constitutes a phytochemical screening tool of high flux.

High-speed counter-current chromatography has drastically improved the efficiency of natural product isolation by liquid–liquid partition [14]. Important strides have been made to define a two-phase solvent system with broad applicability [26]. However, the choice of optimal system is still much contingent upon the chemical nature of the target compound. This was illustrated by the difficulty in resolving $R_F = 0.8$ components of fraction green when the isolation conditions for $R_F = 0.6$ compound were applied. The current studies demonstrate that an efficient CCC isolation requires a judicious combination of several optimized systems, as it became apparent that the initial hexane solvent system and the new chloroform system were more complementary than exclusive. This optimization resulted in a twelve-fold increase in the yield of the major anti-HIV compound mal.4.

A total of 16 lignans of *L. tridentata* were identified in our study [21]. Eight of these compounds are structurally new. Although previously described [5], the lignan 3′-O-methyl NDGA (mal.4) is now made known for its anti-HIV activity from our studies [14]. New lignans, which eluded the classic chromatographic methods were also isolated in the present study.

Mal.4, the major constituent isolated from this plant, was shown to exert its inhibitory activity by interfering with the binding of Sp1 protein to HIV LTR, thus blocking the proviral transcription, Tat transactivation, and suppressing viral replication [14]. Interestingly, these studies revealed that mal.4 acts at Tat-sensitive as well as Tat-insensitive, promoter-containing Sp1 binding sites such as those of SV40 and herpes viruses [14]. Therefore, this class of lignans, in general, may possess a broader antiviral action of important clinical interest.

Acknowledgements

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