Evaluation of Natural Products as Inhibitors of Human Immunodeficiency Virus Type 1 (HIV-1) Reverse Transcriptase

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J. Nat. Prod., **1991**, 54 (1), 143-154• DOI: 10.1021/np50073a012 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 54, No. 1, pp. 143-154, Jan-Feb 1991

EVALUATION OF NATURAL PRODUCTS AS INHIBITORS OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) REVERSE TRANSCRIPTASE[1]

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ABSTRACT.—Inhibition of human immunodeficiency virus reverse transcriptase is currently considered a useful approach in the prophylaxis and intervention of acquired immunodeficiency syndrome (AIDS), and natural products have not been extensively explored as inhibitors of this enzyme. We currently report that the reverse transcriptase assay developed for the detection of the enzyme in virions involving polyadenylic acid·oligodeoxythymidylic acid (poly rA·oligo dT) and radiolabeled thymidine 5′-triphosphate (TTP), can be applied as a simple method for screening the human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) inhibitory potential of natural products. As reported herein, 156 pure natural products have been examined in this system. Benzophenanthridine alkaloids such as fagaronine chloride [**1**] and nitidine chloride, which are known inhibitors of avian myeloblastosis virus reverse transcriptase, demonstrated potent activity in the HIV-1 RT system, and **1** (IC_{50} 10 μg/ml) was adopted as a positive-control substance. Additional inhibitors found were columbamine iodide [**2**] and other protoberberine alkaloids, the isoquinoline alkaloid *O*-methylpsychotrine sulfate [**3**], and the iridoid fulvoplumierin [**4**]. A number of indolizidine, pyrrolizidine, quinolizidine, indole, and other alkaloids, as well as compounds of many other structural classes, were tested and found to be inactive. A total of 100 plant extracts have also been evaluated, and 15 of these extracts showed significant inhibitory activity. Because tannins and other polyphenolic compounds are potent reverse transcriptase inhibitors, methods were evaluated for the removal of these from plant extracts prior to testing. Polyphenolic compounds were found to be responsible for the activity demonstrated by the majority of plant extracts. After appropriate tannin removal procedures were established, the bioassay system was shown to be generally applicable to both pure natural products and plant extracts. The method also proved useful in directing an isolation procedure with *Plumeria rubra* to yield fulvoplumierin [**4**] as an active compound (IC_{50} 45 μg/ml).

The discovery of reverse transcriptase (RT) in RNA tumor viruses demonstrated that genetic information can be transferred from RNA to DNA. The linear DNA product can then be integrated into the host genome. The RT is required for viral replication, and specific inhibitors of RT are useful antiviral agents (1).

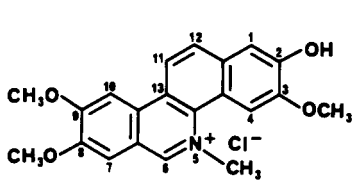
In establishing in vitro systems for screening antiviral agents, RT from RNA tumor viruses has been used by several workers to screen natural products for anti-RT activity. As a result, the benzophenanthridine and protoberberine alkaloids and certain antibiotics were shown to be potent inhibitors of the avian retrovirus RT (2–6). However, interest has shifted to HIV RT since the recognition of the causative agent of acquired immunedeficiency syndrome (AIDS) in 1981. The complex replication cycle of the human immunodeficiency virus in its host cell provides a wealth of target points for the action of drugs, including HIV RT, which is unique to the virus and is critical for viral replication. Because viral replication is essential for progression of AIDS, HIV RT repre-

[1]This paper was presented, in part, at the International Joint Symposium entitled "Biology and Chemistry of Active Natural Substances" held in Bonn, Germany, July 17–22, 1990.

sents one of the most important targets for the development of selective antiviral compounds (7). HIV RT inhibitors such as azidothymidine (AZT), phosphonoformate (PFA), suramin, antimoniotungstate (HPA-23), and 2′,3′-dideoxynucleosides have been used for pharmacological intervention of HIV, even though there are many accompanying toxicities (8). Therefore, it appears worthwhile to search for specific inhibitors of HIV RT that could demonstrate a greater therapeutic index. In addition, novel inhibitors of this enzyme may function by unique mechanisms of action.

The HIV RT assay developed for the detection of the enzyme in virions (9), using polyadenylic acid (poly A), oligodeoxythymidylic acid (oligo dT), and radiolabeled thymidine triphosphate (TTP), can be applied as a simple method for screening the HIV RT inhibitory potential of natural products. The recent availability of purified recombinant HIV-1 RT (10) makes large-scale screening of natural products for HIV RT inhibitory activity feasible. As yet, very few natural products have been shown to inhibit HIV RT specifically. Active compounds, if not eventually advocated for the treatment or prophylaxis of AIDS per se, may serve as new leads for the synthetic development of selective retroviral inhibitors.

Herein we report the development of a bioassay for the screening of natural products and plant extracts for HIV-1 RT inhibitory activity. This includes the establishment of a protocol and definitions of concentration limits for activity, solvent selection, application of the bioassay to an activity-guided fractionation procedure, and the development of strategies involved in the general screening of plant extracts. The method has also been evaluated in terms of sensitivity and selectivity.



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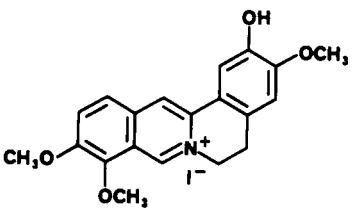

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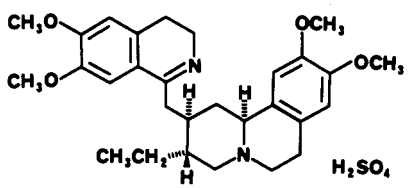

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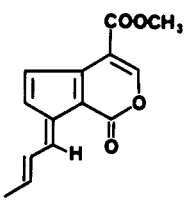

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EXPERIMENTAL

REAGENTS AND TEMPLATES.—[^{3}H]TTP (15 Ci/mmol) was obtained from ICN Radiochemicals (Irvine, California). TTP, poly A, oligo $(dT)_{12-18}$, dithiothreitol, glutathione, gelatin, and soluble polyvinylpyrrolidone (PVP, average mol wt 360,000) were purchased from Sigma Chemical (St. Louis, Missouri). DEAE-cellulose filter discs (Whatman DE 81) were obtained from VWR Scientific (Batavia, Illinois). Polyamide-CC6 powder was obtained from Brinkmann Instruments (Westbury, New York). All other reagents were of analytical grade.

HIV-1 REVERSE TRANSCRIPTASE (HIV-1 RT).—The HIV-1 RT is a 66 kD recombinant enzyme obtained in an *Escherichia coli* expression system using a genetically engineered plasmid; the enzyme was purified to near homogeneity (10). Synthetic DNA segments were used to introduce initiation and termi-

nation codons into the HIV-1 RT coding sequence, and this permitted expression of large quantities of HIV-1 RT in *E. coli* (11). The enzyme was shown to be active in RT assays and exhibited inhibitory properties with several known antiretroviral agents (e.g., AZT and suramin) that are indistinguishable from the viral enzyme (12). The purified recombinant enzyme was sufficiently similar to the viral enzyme that it can be substituted for the latter in drug screening assays (10). The recombinant HIV-1 RT preparation used in all experiments had a protein concentration of 0.11 mg/ml and an activity of 238 nmol TTP incorporated per 10 min per mg protein at 37°. Prior to performing an experiment, the enzyme was diluted tenfold with buffer analogous to that used in the assay.

HIV-1 RT ASSAY.—The assay mixture (final volume 100 μl) contained the following: 50 mM Tris·HCl buffer (pH 8.0), 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM ethylene glyco-bis-(β-aminoethylether) *N,N'*-tetraacetic acid (EGTA), 5 mM dithiothreitol, 0.3 mM glutathione, 2.5 μg/ml bovine serum albumin, 41 μM poly A [ϵ_{260} (mM) = 7.8], 9.5 μM oligo $(dT)_{12-18}$ [Σ_{265} (mM) = 5.6], 0.05% Triton X-100, 20 μM TTP, and 0.5 μCi of [^{3}H]TTP. The reaction was started by the addition of 10 μl of HIV-1 RT, and the mixture was permitted to incubate at 37° for 1 h. Reactions were terminated by the addition of 25 μl of 0.1 M EGTA followed by chilling in ice. Aliquots of each reaction mixture (100 μl) were then spotted uniformly onto circular 2.5 cm DE-81 (Whatman) filters, kept at ambient temperature for 15 min, and washed four times with 5% aqueous $Na_2HPO_4 \cdot 7H_2O$. This was followed by two more washings with doubly distilled H_2O. Finally, the filters were thoroughly dried and subjected to scintillation counting in a nonaqueous scintillation fluid.

For testing enzyme inhibition, five serial dilutions of samples in DMSO (10 μl) were added to the reaction mixtures prior to the addition of enzyme (10 μl). The final DMSO concentration used was 10%. The highest concentration of pure natural products and plant extracts tested was 200 μg/ml. Control assays were performed without the compounds or extracts, but an equivalent volume of DMSO was added. Fagaronine chloride [**1**] was used as the positive control substance. This compound was isolated from *Fagara xanthoxyloides* Lam. (13). Other positive control substances used were suramin (IC_{50} 18 μg/ml) and daunomycin (IC_{50} 125 μg/ml). The assay procedure and the concentration of all components were the same as that mentioned above.

SENSITIVITY OF THE HIV-1 RT ASSAY IN THE PRESENCE OF PLANT EXTRACTS.—Five concentrations of **1** (0.2–25.0 μg/ml) in DMSO were tested in the presence and absence of 100 μg/ml of an extract derived from *Viola yedoensis* Makino. The lowest concentration of **1** was also tested in the presence of 200 μg/ml of *V. yedoensis* extract. Additionally, various amounts of **1** and a constant amount of plant extract were added to each reaction mixture to simulate a final composition of 0.2%, 0.1%, 0.01%, and 0.001% (w/w) active constituent in 200 μg/ml plant extract. This plant species was chosen because in preliminary studies it was devoid of HIV-1 RT inhibitory activity.

BIOACTIVITY-GUIDED FRACTIONATION PROCEDURE.—The milled bark of *Plumeria rubra* L. (Apocynaceae, 10 g) was extracted with petroleum ether (100 ml), after which it was evaporated to dryness below 40° (extract A, 0.17 g). The marc was further extracted with MeOH (100 ml), and the dried MeOH extract (extract B, 1.8 g) was then partitioned between $CHCl_3$-H_2O (1:1). The $CHCl_3$ (extract C) and H_2O (extract D) portions were evaporated to dryness in vacuo to yield residues of 1.3 and 0.16 g, respectively. The dried extracts A, B, C, and D were assayed at a concentration of 200 μg/ml, of which only extract A showed detectable inhibitory activity (35%). Extract A was chromatographed over Si gel (5 g) and eluted with $CHCl_3$-petroleum ether (1:1) to afford an active fraction (520 μg) which showed 70% inhibition of HIV-1 RT activity at 200 μg/ml. Fulvoplumierin [**4**] in this impure fraction was identified by co-tlc in three solvent systems using an authentic sample. The identity of the pure compound obtained from this fraction was confirmed by ms to be **4** (14) which has an IC_{50} value of 45 μg/ml.

POLYAMIDE COLUMN CHROMATOGRAPHY FOR TANNIN REMOVAL.—The general procedure employed was previously established by Wall *et al.* (15). Each plant extract (3 mg) was dissolved in a minimum volume of H_2O and applied to a glass column (0.6 × 10 cm) packed with polyamide powder (400 mg) that had been soaked in H_2O overnight. Elution was performed with H_2O (2 ml) followed by 50% MeOH (2 ml) and finally absolute MeOH (5 ml). Eluate was collected, combined, and evaporated to dryness.

PRECIPITATION OF CONTAMINATING TANNINS WITH GELATIN/NaCl SOLUTION, SOLUBLE POLYVINYLPYRROLIDONE (PVP), AND CAFFEINE.—Gelatin-NaCl solution [containing 5% (w/v) NaCl and 0.5% (w/v) gelatin] is a reagent commonly used for the qualitative detection of tannins in plant extracts (16). Tannin removal methods using soluble PVP and caffeine were conducted according to the general procedure established by Loomis and Battaile (17) and Wall *et al.* (15), respectively. Portions of each dried plant extract (2 mg) found to contain high concentration levels of tannins were dissolved in boiling H_2O (500 μl). Soluble PVP (500 μl) at concentrations between 0 and 10% (w/v) was then added, and the

mixtures were vortexed and centrifuged. Similarly, gelatin/NaCl solution (500 μl) [containing 5% (w/v) NaCl and 0 to 10% (w/v) gelatin] and caffeine at concentrations between 0 and 10% (w/v) were also employed. The final tannin removal concentrations of these agents that resulted in the plant extracts were 0–5% (w/v) (0–50 mg/ml). The aqueous supernatant (2 mg plant extract/ml) was then tested as such for HIV-1 RT inhibitory activity yielding a reaction mixture concentration of 200 μg/ml. The complete removal of tannins was assumed when the percent inhibition of the plant extract became constant as the concentration of PVP, gelatin, or caffeine was increased, or when insignificant inhibitory activity could be detected. Assays were also performed with varying concentrations of PVP, gelatin/NaCl solution, or caffeine to test the individual effect of these reagents on the reaction system. The plant extracts involved in all tannin removal procedures were derived from *Duabanga sonneratioides* Buch.-Ham., *Bridelia retusa* Spreng., *Gardenia coronaria* Buch.-Ham., *Careya sphaerica* Roxb., *Terminalia catappa* L., and *Terminalia alata* Heyne ex Roth. All these extracts were determined in a preliminary study to be rich in tannins using the gelatin-NaCl test.

RESULTS AND DISCUSSION

Preliminary work involved in the development of an RT bioassay included the evaluation of various experimental parameters. The effect of varying enzyme concentrations on thymidine 5′-triphosphate (TTP) incorporation was investigated. As shown in Figure 1, product formation increased in a linear manner throughout the assay period. Based on these incorporation rates, a 1 h incubation time was adopted for these experiments. Figure 2 shows that the rate of incorporation was a linear function of enzyme concentration. Thus, a 10 μl volume (0.11 μg of pure HIV-1 RT) was established as a suitable amount of enzyme to use in each reaction mixture.

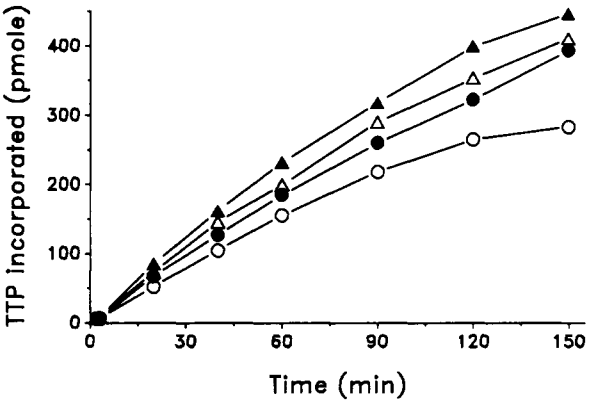

FIGURE 1. Relationship between amount of TTP incorporated and incubation time in the presence of different amounts of HIV-1 RT at 37°. Reaction mixtures were incubated with 10 (○), 12 (●), 14 (△) or 16 (▲) μl of enzyme. Aliquots (100 μl) were withdrawn at the indicated time intervals (0–150 min) and added to 25 μl of EGTA to terminate the enzyme reaction. Data points plotted are the average values obtained from duplicate experiments.

Solvents were found to exert different degrees of inhibitory activity on the polymerization reaction. As shown in Figure 3, the level of inhibition increased with increasing concentrations of DMSO, MeOH, or EtOH. These experiments demonstrated that DMSO at a 10% concentration inhibited the reaction by about 30%, whereas MeOH and EtOH at this concentration were much more inhibitory. Based on these data, DMSO (10% concentration) was selected as the solvent of choice. At this concentration, the solvent was satisfactory for the dissolution of all the test compounds used in the study.

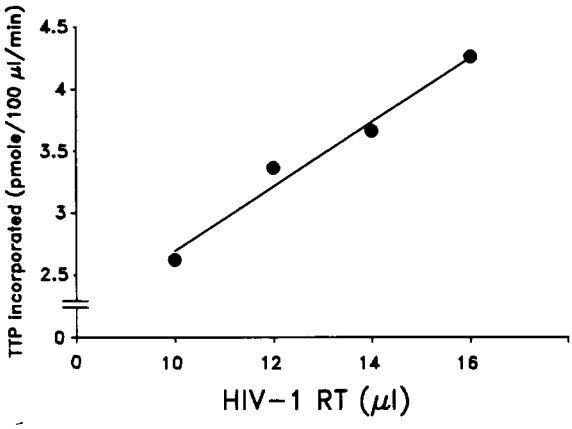

FIGURE 2. Relationship between the initial rate of incorporation (V_o) and the amount of enzyme.

The assay procedure was found to be equally sensitive in detecting the benzophenanthridine alkaloid fagaronine chloride [**1**] in the presence and absence of up to 200 µg/ml of plant extract. Thus, the percent inhibition values obtained for each concentration of **1** tested (0.2–25.0 µg/ml) and the IC_{50} values calculated were identical in both cases (data not shown). The lower limit of detection was found to be 0.02 µg/ml of **1** per 200 µg/ml of extract (i.e., 0.01% active constituent in the plant extract).

A prescreen with only one concentration of pure compound or extract (200 µg/ml) was found to be sufficient to detect active compounds or extracts. The IC_{50} values of all active compounds and extracts were then determined by testing five concentrations in duplicate.

Pure compounds representing many natural product classes were tested. A number of benzophenanthridine alkaloids such as **1** and nitidine chloride have shown significant cytotoxic activity against P-388 leukemia in mice (13,18). These compounds have also been reported to be effective inhibitors of the mouse DNA polymerase and of the RTs of avian myeloblastosis virus (AMV), Rauscher murine leukemia virus, and simian sarcoma virus (19). As shown in Figure 4, **1** (IC_{50} 10 µg/ml) and nitidine chloride (IC_{50}

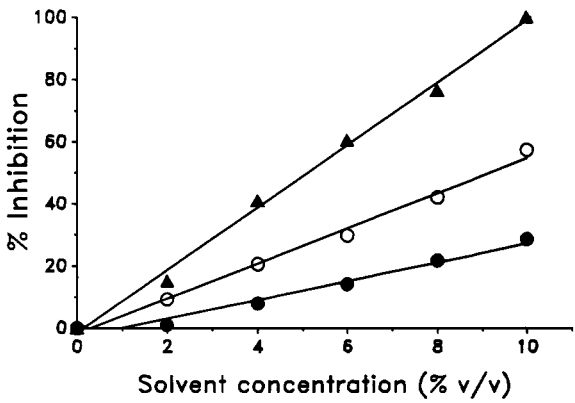

FIGURE 3. Effect of solvents on HIV-1 RT activity. HIV-1 RT activity was determined as described in Experimental except that the indicated concentrations of DMSO (●), MeOH (○), or EtOH (▲) were added to the incubation mixtures.

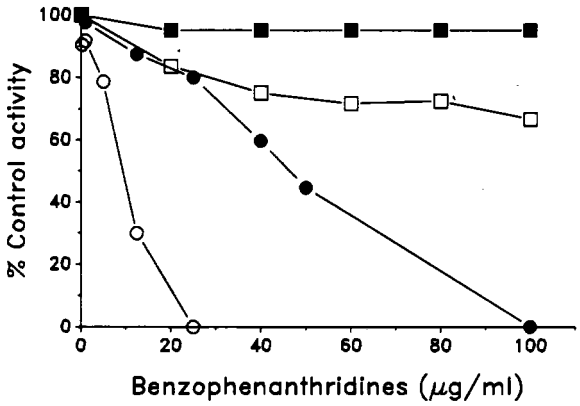

FIGURE 4. Effect of benzophenanthridine concentration on HIV-1 RT activity with poly A·oligo $(dT)_{12-18}$ as template-primer. Five serial dilutions of samples in DMSO (10 μl) were added to the reaction mixture just prior to incubation. Enzyme preparation (10 μl) was then added to the incubated tubes at regular time intervals. Control assays were performed without the compounds or extracts but contained an equivalent volume of DMSO. Results were expressed as % control activity. Representative alkaloids tested were **1** (○), nitidine chloride (●), chelidonine (□), and *N*-demethylfagaronine (■).

49 μg/ml) were observed to be potent HIV-1 RT inhibitors. Chelidonine had an IC_{50} of about 200 μg/ml and was considered weakly active, while *N*-demethylfagaronine was inactive (<10% inhibition at 200 μg/ml). Observations on the structure-activity relationships of benzophenanthridine alkaloids were analogous to those published for AMV RT (5). A hydroxyl group at position 2 enhanced activity within the class, and the optimum positions for the attachment of methoxy groups were C-8 and C-9, rather than C-7 and C-8. The quaternary nitrogen is believed to be important for anchoring such alkaloids to the double-stranded nucleic acid template (20). Although this functionality is required for the inhibitory activity of benzophenanthridine alkaloids, it is not solely responsible because quaternary alkaloids of other structural classes, e.g., echitamine chloride (an indole alkaloid) and magnoflorine chloride (an aporphine), were virtually inactive (<10% inhibition at 200 μg/ml).

The protoberberine alkaloids are structurally related to the benzophenanthridines and have been shown to inhibit AMV RT (4). They have also been shown to possess a wide variety of biological properties such as antimicrobial and anticancer activities (21). Columbamine iodide [**2**] (IC_{50} 58 μg/ml), coptisine picrate (IC_{50} 56 μg/ml), jatrorrhizine chloride (IC_{50} 71 μg/ml), and berberine chloride (IC_{50} 100 μg/ml) were found to be moderately potent, whereas compounds that lacked the quaternary nitrogen, e.g., tetrahydropalmatine and tetrahydroberberine, were inactive (25% inhibition at 200 μg/ml). The unavailability of more diversified analogues of the protoberberines prevented significant structure-activity correlation in this series of alkaloids.

The benzophenanthridine and protoberberine alkaloids are derived biosynthetically from benzylisoquinoline intermediates. The majority of alkaloids with this biosynthetic origin, however, retain the isoquinoline ring structure, as exemplified by the alkaloids from ipecac, the dried rhizome and roots of *Cephaelis ipecacuanha* (Brotero) A. Richard (Rubiaceae). Ipecac yields more than 2% w/w of Et_2O-soluble alkaloids, with

the three principal ones being emetine, cephaeline, and psychotrine (22) and with *O*-methyl-psychotrine constituting one of the minor alkaloids (23). These structurally related alkaloids are prepared as their inorganic acid salts for solubility and stability reasons. Their pharmacological effects include antiamebic, antitumor, and antiviral activities, in addition to the irreversible inhibition of DNA synthesis (23). They also inhibit protein synthesis by inhibiting peptide elongation at the stage of translocation (23). While emetine hydrochloride, cephaeline dihydrochloride, and psychotrine free base were not active in inhibiting the HIV-1 RT (ca. 20% inhibition at 200 μg/ml), *O*-methyl-psychotrine sulfate [3] had an IC_{50} value of 10 μg/ml (data not shown).

Flavonoids like tiliroside, quercetin, and kaempferol were found to be weak HIV-1 RT inhibitors (IC_{50} 150–200 μg/ml). This could be due to their weak intercalating properties, which are enhanced by structural planarity (24). Gossypol acetic acid, an anti-HIV compound (25), also showed weak inhibitory activity (IC_{50} 170 μg/ml). It is possible that this activity was due to the presence of its several phenolic hydroxy groups, because these substituents have been linked with HIV RT inhibitory activity (6).

Representatives of the quinolizidine (e.g., ormosinine and sparteine), pyrrolizidine (e.g., retrorsine) and indolizidine (e.g., swainsonine) alkaloidal groups were tested and found to be inactive (IC_{50}>200 μg/ml). The HIV-1 RT inhibitory potential of acronycine and its derivatives (acridone alkaloids) could not be accurately assessed due to their low solubility in H_2O, resulting in precipitation in the aqueous reaction mixture. Some of the other inactive natural products tested belonged to the following classes: limonoids, lignans, sterols, quinones, triterpene glycosides, sesquiterpenes, saponin glycosides, steroidal cardiac glycosides, coumarins, rotenoids, and alkaloids belonging to the quinoline, benzylisoquinoline, aporphine, and indole types. Certain highly cytotoxic compounds were found to lack HIV-1 RT inhibitory activity, e.g., taxol (26) and didemnin B (27). Equisetin, a *Fusarium* toxin (28), was inactive despite its structural resemblance to a nucleoside.

In total, 156 compounds were tested (Table 1). A numerical summary of the results is as follows: four were active (IC_{50}<50 μg/ml); twelve moderately active (50 μg/ml <IC_{50}<150 μg/ml); five weakly active (150 μg/ml<IC_{50}<200 μg/ml); and 135 inactive (IC_{50}>200 μg/ml).

TABLE 1. Compounds Tested for HIV-1 RT Inhibitory Activity.[a]

Acridone alkaloids: acronycine (IA), acronycine azine (IA), dihydroacronycine (IA), nitroacronycine (IA), thioacronycine (IA)
Alkaloidal amine: colchicine (IA), ephedrine sulfate (IA), phenylephrine (IA)
Ansa macrolide: maytansine (IA)
Anthracycline antibiotic: daunomycin (MA)
Aporphine alkaloids: boldine (IA), magnoflorine chloride (IA), melosmine (IA)
Benzophenanthridine alkaloids: chelerythrine chloride (WA), chelidonine (WA), *N*-demethylfagaronine (IA), fagaronine chloride (A), nitidine chloride (A), sanguinarine nitrate (MA)
Cardiac glycosides: ouabain (IA)
Cephalotaxus alkaloids: harringtonine (IA), homoharringtonine (IA)
Chromone: khellin (IA)
Coumarins: esculin (IA), thamnosmonin (IA)
Diterpene alkaloids: cassaine sulfate (IA), taxol (IA)
Diterpenes/diterpene glycosides: steviol (IA), stevioside (IA)
Flavonoids: 2′,4-dihydrochalcone (IA), 8-methoxy-5,7-dihydroflavonol (IA), genkwanin (IA), kaempferol (MA), quercetin (WA), tiliroside (WA), velutin (IA)
Furocoumarin: 5-(3′-methyl-2′,3′-dihydroxybutyl)-8-methoxy psoralen (IA)
Furoquinoline: acetylrobustine (IA), heliparvifoline (IA)
Imidazole alkaloid: isopilosine (IA)

Indole alkaloids: ajmalicine (IA), ajmaline (IA), alstonine hydrochloride (MA), aspidospermine (IA), aricine (IA), brucine (IA), camptothecine (IA), catharanthine hydrochloride (IA), conoflorine (IA), conopharyngine (IA), corynantheidine (IA), corynantheine (IA), 1,2-dehydroaspidospermine (IA), deserpideine hydrochloride (IA), deserpidine (IA), echitamine chloride (IA), ellipticine (MA), isoschizogamine (IA), isoraunescine (IA), leurosine sulfate (IA), lochnerine (IA), *N*-acetylperivine (IA), narceine (IA), *O*-methylellipticine (MA), perakine (IA), pericalline (IA), pericyclivine (IA), periformyline (IA), perividine (IA), perivine (IA), rauniticine (IA), raunitidine (IA), sarpagine (IA), schizozygine (IA), tetrahydroalstonine (IA), tubulosine (IA), vinblastine (IA), vincamajine (IA), vincristine (IA)

Indolizidine alkaloid: swainsonine (IA)

Iridoids: 13-*O*-*t*-coumaroylplumieride (IA), 15-demethylplumieride (IA), fulvoplumierin (A), plumericin (IA), plumieride (IA)

Isoquinoline alkaloids: cephaeline dihydrochloride (IA), emetine hydrochloride (IA), hernandezine (IA), hydrastine (IA), *O*-methylpsychotrine sulfate (A), papaverine (WA), psychotrine (IA), thalicarpine (IA), tubocurarine chloride (IA)

Lignan: liriodendrin (IA)

Limonoids: limonin (IA), nomilin (IA)

Macrocyclic peptide: didemnin B (IA)

Piperidine alkaloids: arecoline hydrobromide (IA), trigonelline hydrochloride (IA)

Polyphenolics: ellagic acid (MA), tannic acid (A)

Protoberberine alkaloids: berberine chloride (MA), canadine methchloride (IA), columbamine iodide (MA), coptisine chloride (MA), dihydroberberine (IA), jatrorrhizine chloride (MA), tetrahydroberberine (IA), tetrahydropalmatine (IA), tetrahydropalmatrubine (IA)

γ-Pyrone: *O*-methylkojic acid (IA)

Pyrrolizidine alkaloids: indicine *N*-oxide (IA), lasiocarpine (IA), monocrotaline (IA), retrorsine (IA), senecionine (IA), seneciphylline (IA)

Quinoline alkaloids: cinchonine (IA), quinidine sulfate (IA)

Quassinoid: bruceantin (IA)

Quinolizidine alkaloids: nesodine (IA), ormosinine (IA), panamine (IA), panamine diperchlorate (IA), sparteine (IA), sparteine sulfate (IA), leontine (IA)

Quinones/quinols: 2,5-dimethyl benzoquinone (IA), 3-tert-butyl-4-methoxy-1,6-dione (IA), jacaranone (IA)

Rotenoids: 12-hydroxypachyrrhizone (IA), 12a-hydroxyrotenone (IA), rotenone (IA)

Sesquiterpenes/sesquiterpene lactones: gossypol acetic acid (MA), picrotoxin (IA)

Sesquiterpene alkaloid: phyllanthoside (IA)

Steroids/steroidal glycosides: δ-4-androsten-3,17-dione (IA), 4β-hydroxywithanolide (IA), polypodin (IA)

Steroidal alkaloids: tomatine (IA), veratrine (IA)

Sterol: stigmasterol (IA)

Sulfated polysaccharide: sodium heparin (IA)

Triterpenes: β-amyrin (IA), aridanin (IA), betulin (IA), 22-hydroxytingenone (IA), betulin (IA), primulagenin (IA)

Triterpene saponin glycosides: ginsenoside Rb_1 (IA), ginsenoside Re (IA)

Tropane alkaloids: hyoscine hydrobromide (IA), scopolamine hydrobromide (IA)

[a]A = Active, MA = Moderately Active, WA = Weakly Active, IA = Inactive.

Approximately 100 crude EtOH or MeOH extracts from a diverse group of plant families were also tested (Table 2). Included were several which have been shown by in vitro tests to demonstrate anti-HIV activity (29), namely, *Arctium lappa* L., *Viola yedoensis* Makino, *Lithospermum erythrorhizon* Sieb. & Zucc., *Coptis chinensis* Franch., *Lonicera japonica* Thunb., *Epimedium grandiflorum* Morr., and *Prunella vulgaris* L. Of these, *C. chinensis* (IC_{50} 108 μg/ml) was found to be significantly active. Of the 100 extracts evaluated, 15 showed >50% inhibition at 200 μg/ml and were therefore consid-

TABLE 2. Plant Extracts Tested for HIV-1 RT Inhibitory Activity.[a]

Acanthaceae: *Ruellia tuberosa* L. (16%), *Acanthus ilicifolius* L. (1%)

Amaranthaceae: *Amaranthus tricolor* L. (10%), *Amaranthus spinosus* L. (11%)

Annonaceae: *Artabotrys siamensis* Miq. (MeOH) (24%), *Desmos chinensis* Lour. (17%), *Melodorum fruticosum* Lour. (MeOH) (18%), *Miliusa velutina* Hook. f. & Th. (11%), *Rauwenhoffia siamensis* Scheff. (11%)
Apocynaceae: *Allamanda violaceae* Gard. & Field (22%), *Beaumontia brevituba* Oliv. (8%), *Wrightia religiosa* Benth. (8%), *Alstonia macrophylla* Wall. (9%), *Wrightia tomentosa* Roem. & Schult. (10%)
Averrhoaceae: *Averrhoa carambola* L. (12%)
Avicenniaceae: *Avicennia alba* Bl. (14%), *Avicennia officinalis* L. (5%)
Barringtoniaceae: *Careya sphaerica* Roxb. (75%)
Berberidaceae: *Epimedium grandiflorum* Morr. (15%) (IC_{50} = 580 μg/ml)
Bignoniaceae: *Tabebuia rosea* DC. (24%), *Millingtonia hortensis* Linn. f. (21%), *Markhamia stipulata* Seem. (MeOH) (18%), *Fernandoa adenophylla* Steenis (16%), *Radermachera ignea* Steenis (12%), *Stereospermum personatum* Chatterjee (17%), *Pachyptera hymenaea* A. Gentry (9%), *Dolichandrone spathacea* Schum. (8%)
Bombacaceae: *Bombax ceiba* L. (23%), *Ceiba pentandra* Gaertn. (18%)
Boraginaceae: *Lithospermum erythrorhizon* Sieb. & Zucc. (60%*) (IC_{50} = 212 μg/ml)
Capparidaceae: *Cleome viscosa* L. (8%)
Caprifoliaceae: *Lonicera japonica* Thunb. (0%) (IC_{50} = 1021 μg/ml)
Casuarinaceae: *Casuarina junghuhniana* Miq. (48%)
Celastraceae: *Siphonodon celastrineus* Griff. (MeOH) (6%)
Combretaceae: *Terminalia catappa* L. (97%*), *Terminalia alata* Heyne ex Roth (90%*)
Compositae: *Blumea balsamifera* DC. (20%)
Cucurbitaceae: *Coccinia grandis* Voigt (10%), *Luffa cylindrica* Roem. (12%)
Cyperaceae: *Fimbristylis quinquangularis* Kunth (0%)
Dipterocarpaceae: *Shorea obtusa* Wall. (12%)
Ebenaceae: *Diospyros mollis* Griff. (MeOH) (11%), *Diospyros gracilis* Fletch. (MeOH) (30%), *Diospyros montana* Roxb. (MeOH) (16%), *Diospyros rhodocalyx* Kurz (9.4%)
Elaeocarpaceae: *Muntingia calabura* L. (21%)
Euphorbiaceae: *Aporosa villosa* Baill. (60%*), *Sauropus androgynus* Merr. (13%), *Suregada multiflorum* Baill. (7%), *Croton crassifolius* Giesel (23%), *Cladogynos orientalis* Zipp. ex Span. (14%), *Bridelia retusa* Spreng. (45%*), *Breynia angustifolia* Hook. f. (57%*), *Drypetes roxburghii* Wall. (45%*), *Securinega leucopyrus* Muell. Arg. (68%*)
Flacourtiaceae: *Homalium grandiflorum* Benth. (8%), *Homalium tomentosum* Benth. (18%)
Guttiferae: *Calophyllum inophyllum* L. (16%)
Labiatae: *Prunella vulgaris* L. (20%) (IC_{50} = 385 μg/ml)
Lauraceae: *Cinnamomum iners* Bl. (8%)
Lecythidaceae: *Couroupita guianensis* Aubl. (MeOH) (24%)
Leguminosae: *Bauhinia winitii* Craib (11%), *Acacia catechu* Willd. (10%)
Meliaceae: *Aglaia odorata* Lour. (MeOH) (9%), *Sandoricum koetjape* Merr. (11%), *Xylocarpus granatum* Koen. (11%), *Walsura trijuga* Kurz (12%)
Memecylaceae: *Memecylon edule* Roxb. (42%)
Oleaceae: *Myxopyrum smilacifolium* Bl. (22%), *Schrebera swietenioides* Roxb. (53%*)
Palmae: *Salacca conferta* Griff. (5%)
Polygonaceae: *Triplaris cummingiana* Fisch. & Mey. (15%)
Ranunculaceae: *Coptis chinensis* Franch. (94%*) (IC_{50} = 108 μg/ml)
Rhizophoraceae: *Carallia brachiata* Merr. (3%)
Rubiaceae: *Hymenodictyon excelsum* Wall. (19%), *Gardenia coronaria* Buch.-Ham (51%*), *Gardenia sootepensis* Hutch. (28%), *Gardenia erythroclada* Kurz (9%), *Morinda citrifolia* L. (5%)
Rutaceae: *Murraya paniculata* Jack (7%)
Salicaceae: *Salix tetrasperma* Roxb. (17%)
Salvadoraceae: *Azima sarmentosa* Benth. & Hook. (3%)
Sapindaceae: *Paranephelium longifoliolatum* Lec. (45%*), *Schleichera oleosa* Merr. (8%)
Simaroubaceae: *Harrisonia perforata* Merrill (MeOH) (68%*), *Irvingia malayana* Oliv. ex A. Benn. (37%), *Ailanthus triphysa* Alston (19%)
Sonneratiaceae: *Duabanga sonneratioides* Buch.-Ham. (94%*)
Staphyleaceae: *Turpinia cochinchinensis* Merr. (45%*)
Stilaginaceae: *Antidesma sootepense* Craib (15%)
Thymelaeaceae: *Enkleia siamensis* Nervling (20%)
Ulmaceae: *Celtis timorensis* Span. (4%)
Verbenaceae: *Volkameria fragrans* Vent. (27%)
Violaceae: *Viola yedoensis* Makine (16%) (IC_{50} = 615 μg/ml)

[a]% Inhibition values at 200 μg/ml are expressed relative to control assays and are shown in parentheses. All extracts tested were EtOH extracts except when otherwise indicated. Significantly active extracts are marked with an asterisk.

ered significantly active. A response of this magnitude was considered indicative of the presence of high concentrations of potent inhibitory compounds in the plant extract, e.g., berberine (IC_{50} 100 μg/ml) in the roots of *Co. chinensis*. However, tannins, which appear to be very common in the plant kingdom, could also mediate a similar response. Thirteen of the active extracts gave copious precipitates with gelatin/NaCl solution, indicating that they contained high concentrations of tannins, which are known inhibitors of RTs from RNA tumor viruses (6). Also consistent with this suggestion is the fact that commercial samples of tannic and ellagic acids were found to inhibit the HIV-1 RT (80–100% inhibition at 200 μg/ml).

Relative to tannins, most non-polyphenolic inhibitory compounds are present in much lower concentrations in plant extracts. When inhibitors such as **1**, **2**, and jatrorrhizine chloride were tested at concentrations between 0.002 and 0.4 μg/ml (simulating a 0.001% to 0.2% composition in a 200 μg/ml plant extract), only 15 to 20% inhibition was observed. Thus, in the absence of tannins, responses of this magnitude must be considered significant. However, because it is generally not known from the outset whether the inhibition mediated by a plant extract is due to tannins or other substances, chromatographic fractionation of active extracts is required, and inhibition obtained with concentrated fractions will serve as a better indication of the presence of non-tannin inhibitory compounds.

Experiments were also conducted to find quick and simple procedures for tannin removal that would not significantly affect the efficiency of the screening process. As reported previously (15), chromatography on polyamide is effective for the selective removal of polyphenolics. With slight modification, this procedure can be accomplished quickly and conveniently with only a few milligrams of plant extract. It was also observed that non-tannin inhibitory compounds with two or three phenolic hydroxy groups can be eluted by flushing with MeOH.

Precipitation of tannins with gelatin/NaCl solution was found to be an unsuitable method for tannin removal from plant extracts even though it serves as a very simple qualitative test for the presence of tannins (16). Difficulty was encountered in using the procedure quantitatively because the gelatin itself was added as an aqueous solution and there was uncertainty as to the amount to add to the plant extract for the complete precipitation of tannins. Furthermore, the viscosity of gelatin and the excess amounts in solution interfered with the enzyme assay.

Similar problems were encountered when soluble polyvinylpyrrolidone (PVP) (17) was used as a method for tannin removal. The method, however, was moderately successful because of the higher capacity of PVP for binding tannins and its less viscous nature. Percent inhibition values of plant extracts decreased as the concentration of PVP used was increased. It was found that 500 μl of a 10% w/v PVP solution was sufficient for the complete removal of tannins from 2 mg of all the plant extracts tested, which were initially dissolved in 500 μl of H_2O. This corresponds to an effective concentration of 50 mg/ml (5% w/v) PVP to 2 mg/ml of plant extract. When 10 μl of PVP (50 mg/ml) was tested, there was stimulation of enzyme activity. At present, the reason for this stimulation is unknown. Nonetheless, at this stimulatory concentration, PVP did not interfere with the inhibitory effect of test compounds as demonstrated by the identical inhibition values observed in the presence and absence of PVP.

The use of caffeine (15), though free of viscosity problems, introduces a problem of solubility (ca. 20 mg/ml at 20°). A 100 mg/ml (10% w/v) aqueous solution can be readily achieved by heating; however, excess amounts recrystallized upon cooling. Because of the low capacity with which caffeine can bind to tannins, hot saturated caffeine solution (60°) must be used for plant extracts rich in polyphenolics, and 500 μl of a hot 10% w/v caffeine solution was insufficient for the complete removal of tannins from 2 mg of

all the plant extracts involved. It was also determined that the minimum inhibitory concentration of caffeine is approximately 2 mg/ml (2% w/v). Therefore, in cases where excess free caffeine exists in solution, enzyme inhibition may be observed, producing misleading results. The uncertainty as to the precise amount needed for use with each plant extract makes this method unacceptable for tannin removal in the course of sample preparation prior to screening. Also, because $CHCl_3$-soluble components had not been previously removed from the EtOH or MeOH plant extracts, it was not possible to remove the excess caffeine in solution by solvent extraction.

The interaction leading to the removal of tannins is the formation of hydrogen bonds between the tannin phenolic hydroxy groups and the amide link (CONH) of the precipitating agent. This interaction yields insoluble complexes. Therefore, these procedures also remove non-tannin inhibitory compounds with phenolic hydroxy groups (e.g., flavonoids), except those that have consecutive *ortho*-hydroxy groups which are internally hydrogen-bonded (although certain flavonoids could be recovered from a polyamide column by washing with MeOH). Because of this structural characteristic, hydrolyzable tannins have been reported to elude this separation process. Furthermore, quinones are irreversibly bound to these agents due to a covalent interaction (17).

Thus, no single quantitative procedure involving the addition of a precipitating reagent was found to be suitable for tannin removal from plant extracts in preparation for the HIV-1 RT assay, and no generalizations could be made regarding the concentrations or type of reagents that should be adopted. Different plant species contain different types of polyphenolics at highly variable concentrations. In our experience, the method of choice is polyamide cc, which is reliable and which does not introduce any extraneous material to the plant extract to be tested.

When the bioassay was applied at various stages of the fractionation scheme of the bark of *P. rubra*, it proved capable of directing the isolation procedure to yield fulvoplumierin [**4**] from the petroleum ether extract after Si gel cc. This extract showed about 35% inhibition at 200 μg/ml, whereas the aqueous and $CHCl_3$ extracts were devoid of detectable inhibitory activity. Compound **4** demonstrated strong inhibitory activity with an IC_{50} value of 45 μg/ml (data not shown). This led to the evaluation of additional iridoids, none of which was found to be active, including plumericin and plumieride.

In conclusion, the standard reverse transcriptase assay is a specific, sensitive, simple, and reliable method for screening the HIV-1 RT inhibitory potential of natural products. Results are reproducible, and the procedure is applicable to both pure natural products and plant extracts. Moreover, the method has proven to be useful in a bioactivity-guided fractionation procedure. In general, only non-tannin inhibitory compounds are of interest. However, tannins are active inhibitors of reverse transcriptase, and no clear-cut threshold for activity could be established for plant extracts containing tannins. Tannins should be removed before the assay is performed. In the absence of tannins, a percent inhibition value of >50 at a concentration of 200 μg/ml of plant extract (an IC_{50} <200 μg/ml) was selected as indicating potent HIV-1 RT inhibitory activity. However, a 10 to 40% inhibition value may suggest the presence of a single potent inhibitor in very low concentrations. In the case of pure natural products, $>90\%$ inhibition at 200 μg/ml (or IC_{50} <50 μg/ml) was established as the threshold for potent HIV-1 RT inhibitory activity. Compounds showing percent inhibition values between 50 and 90% at 200 μg/ml (or IC_{50} values between 50 and 150 μg/ml), may be classified as moderately potent. Weak inhibitors show between 30 and 50% inhibition at 200 μg/ml (or IC_{50} values between 150 and 200 μg/ml). Potent natural product HIV-1 RT inhibitors discovered using the present protocol include compounds from the benzophenanthridine alkaloid, protoberberine alkaloid, isoquinoline alkaloid, and iridoid classes.

ACKNOWLEDGMENTS

The authors thank Professor K.L. Rinehart of the Department of Chemistry, University of Illinois, Urbana, Illinois, for the sample of didemnin B, Professor D.G. Lynn of the Department of Chemistry, University of Chicago, for a sample of equisetin, and the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, for the provision of nitidine, taxol, maytansine, and vinblastine. We are also grateful to Professor V. Rentrakul, Department of Chemistry, Mahidol University, Thailand, and Professor N.R. Farnsworth of this institution for supplying several of the plant extracts. Additional test compounds were obtained from the compound repository and several individual faculty members at the Program for Collaborative Research in the Pharmaceutical Sciences and Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago. JMP is the recipient of a Research Career Development Award from the National Cancer Institute (1984–1989) and a Fellowship from the Alexander von Humboldt Foundation (1990–1991). GTT is grateful to the American Pharmaceutical Association and the American Society of Pharmacognosy for the award of the Kilmer Prize in recognition of the work described in this paper. We are also grateful to P.K. Clark, D.A. Miller, and M.L. Mellini of Program Resources, Inc., who prepared the HIV-1 RT. Research sponsored in part by the National Cancer Institute, DHHS, under contract No. NO1-CO-74101 with Advanced Bioscience Laboratories, Inc.

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Received 1 June 1990