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A CHEMICAL SCREENING STRATEGY FOR THE DEREPLICATION AND PRIORITIZATION OF HIV-INHIBITORY AQUEOUS NATURAL PRODUCTS EXTRACTS

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ABSTRACT.—A relatively high percentage (ca. 15%) of aqueous extracts from terrestrial plants, cyanobacteria, and marine invertebrates and algae has exhibited activity in the National Cancer Institute's primary AIDS-antiviral screen. By removal of anionic polysaccharides in a first stage of dereplication, we have eliminated from further consideration a considerable number of these extracts. However, a still substantial proportion of the active extracts remained, from which we wished to select and prioritize a small percentage for our detailed bioassay-directed fractionation studies. Therefore, a chemical screening protocol, utilizing various solid-phase extraction cartridges, has been developed for a second-stage dereplication and to assist in prioritization of these extracts for our further investigations.

In late 1987, the National Cancer Institute began an extensive evaluation of natural product extracts derived from microorganisms, plants, and marine invertebrates and algae for HIV-inhibitory activity (1). In the intervening five years, nearly 40,000 crude aqueous and organic solvent extracts have been tested in the primary anti-HIV screen (2). The overall status of the NCI anti-HIV primary screening of crude aqueous extracts through October 1992, is summarized in Table 1. A surprisingly large number (ca. 15%) of the aqueous extracts of terrestrial plants and lichens, cultured cyanobacteria, and marine invertebrates and algae has exhibited some activity in the screen. The high percentage of active extracts strongly suggested the presence of one or more recurring compound classes with HIV-inhibitory activity. In order to help focus our NCI intramural research efforts upon a limited number of extracts, we first developed and are applying a simple precipitation strategy for the initial dereplication (elimination) of extracts containing anti-HIV-active sulfated polysaccharides (3). This procedure has substantially decreased the number of leads retained for further consideration. However, a considerable proportion of the supernatant fractions contain HIV-inhibitory activity not apparently associated with sulfated polysaccharides. Therefore, we sought to develop a protocol for the preliminary general chemical characterization of these AIDS-antiviral aqueous extracts which we could apply to a second stage of dereplication and subsequent prioritization of a practical number of extracts for our further study.

We had been experimenting with solid phase extraction cartridges to scout for fractionation approaches suitable for our aqueous extracts. In so doing, it occurred to us that we might be able to utilize this scouting technique for a preliminary chemical characterization, or "chemical screening," as a follow-up stage of selection from the remaining active extracts. Such an approach would allow us to gain insight into the general chemical nature of potential new leads, to identify and dereplicate additional recurring classes of antiviral compounds, and to select chromatographic procedures for initial fractionation of the remaining extracts of greatest interest to us.

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TABLE 1. Current Anti-HIV Primary Screen Results for Natural Products Extracts.

Organism Group	Total # Extracts Tested[a]	Extracts Selected for Initial LDDRD[b,c] Follow-up		Number Precipitated[c]	Number Active Supernatants[c]
		Organic[d]	Aqueous[e]		
Marine Invertebrates	5,741	74	635	606	74
Marine Plants	1,779	15	307	268	51
Cyanobacteria	1,754	26	124	99	43
Terrestrial Plants	16,886	255	1174	526	303
Lichens	819	11	80	20	6

[a]As of October 31, 1992. Information provided by Dr. Kenneth Snader, Natural Products Branch, DTP, DCT, NCI; each total is divided approximately equally between organic and aqueous extracts.

[b]NCI Laboratory of Drug Discovery Research and Development. Current criteria for selection: $EC_{50}<250$ μg/ml.

[c]As of October 31, 1992. Information summarized from unpublished data from the Laboratory of Drug Discovery Research and Development, DTP, DCT, NCI.

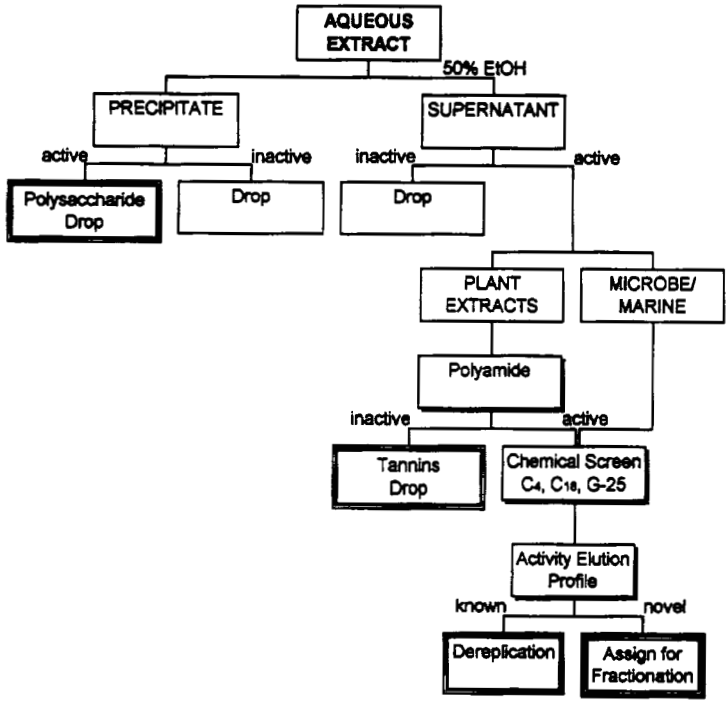
[d]Organic extracts prepared by steeping in CH_2Cl_2-MeOH (1:1) followed by MeOH.

[e]Aqueous extracts prepared by steeping in distilled H_2O.

RESULTS

After evaluating a number of cartridge packings, a standard protocol was defined for all anti-HIV active supernatants remaining under consideration after the initial polysaccharide dereplication procedure (3). Sephadex G-25 cartridges were utilized to provide information about molecular size and weight. Bonded-phase cartridges, C_4 wide pore (300 Å) and C_{18} narrow pore (60 Å), were utilized to determine the relative polarity of active constituents. Distinctive retention differences on wide and narrow pore gels would be informative about molecular size and shape and, in particular, could be indicative of peptides. Four fractions were collected from each cartridge; they were tested side by side with the parent supernatant for anti-HIV activity. Thus, a distinctive or characteristic chromatographic profile of the active constituents(s) was obtained, along with information about the recovery of activity and, by inference, the stability of the active compound(s). The percentage of HIV-inhibitory supernatants has been highest among plant extracts; this observation, coupled with a number of recent reports of the HIV-inhibitory properties of tannins (4–7), suggested that a simple procedure to identify tannin-containing extracts would also be useful. Therefore, an additional cartridge containing polyamide resin was utilized to screen plant extracts for tannins; polyphenolics have long been known to be irreversibly retained on polyamide resins (8), while compounds with fewer phenolic residues (e.g., flavonoids) can be eluted from polyamide gels with MeOH (7). This extra step for plant extracts occurs prior to the standard chemical screen; in this manner, the number of fractions produced overall was reduced for tannin-containing extracts. Scheme 1 illustrates our overall dereplication and chemical screening strategy.

The validity of this approach as a chemical screen was investigated with a number of HIV-inhibitory standards, e.g., AZT, dextran sulfate, cyclosporin, and oxathiin carboxanilide (9). As illustrated in Figure 1, different patterns of elution were observed for each compound tested. Presented in the matrix form shown, recurring patterns of bioactivity elution could be readily discerned, as was the case for a series of sponge extracts containing sulfated sterols. Several sponge extracts revealed a novel but consistent pattern of elution in the chemical screen (Figure 2). Nmr analyses suggested that each contained sulfated sterols; complete details of the structures and biological activity of our isolates will be presented elsewhere (10; T.C. McKee, J.H. Cardellina II, J.B. McMahon, L. Minale, R. Riccio, K.M. Snader, and M.R. Boyd, manuscript in preparation). HIV-inhibitory activity has recently been reported for this class (10–12).



SCHEME 1

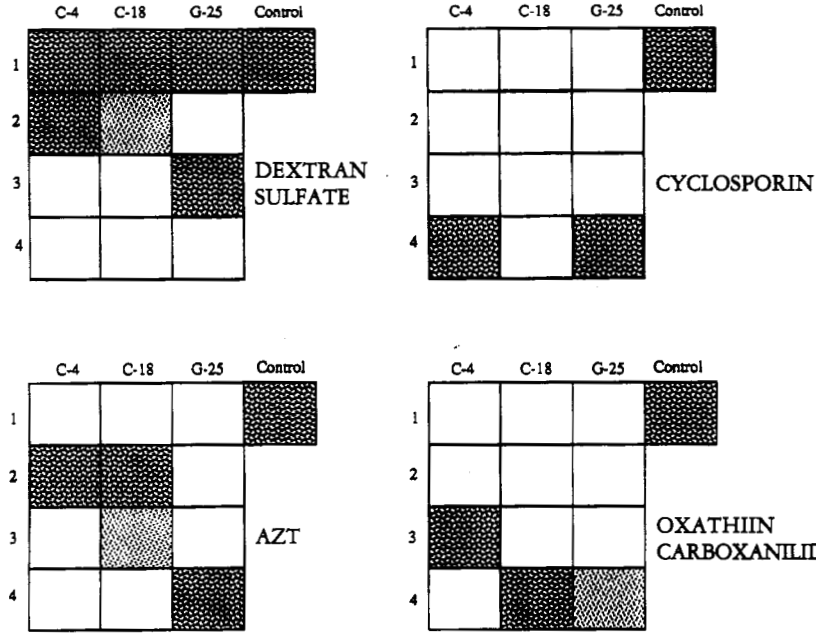


FIGURE 1. Chemical screening patterns for HIV-inhibitory standards. The 3×4 matrix represents four fractions each from three different chromatography media. Heavily shaded boxes indicate elution of anti-HIV activity comparable to a control sample of the standard antiviral agent. More lightly shaded boxes indicate elution of activity of lower potency than the control; open boxes indicate no elution of anti-HIV activity.

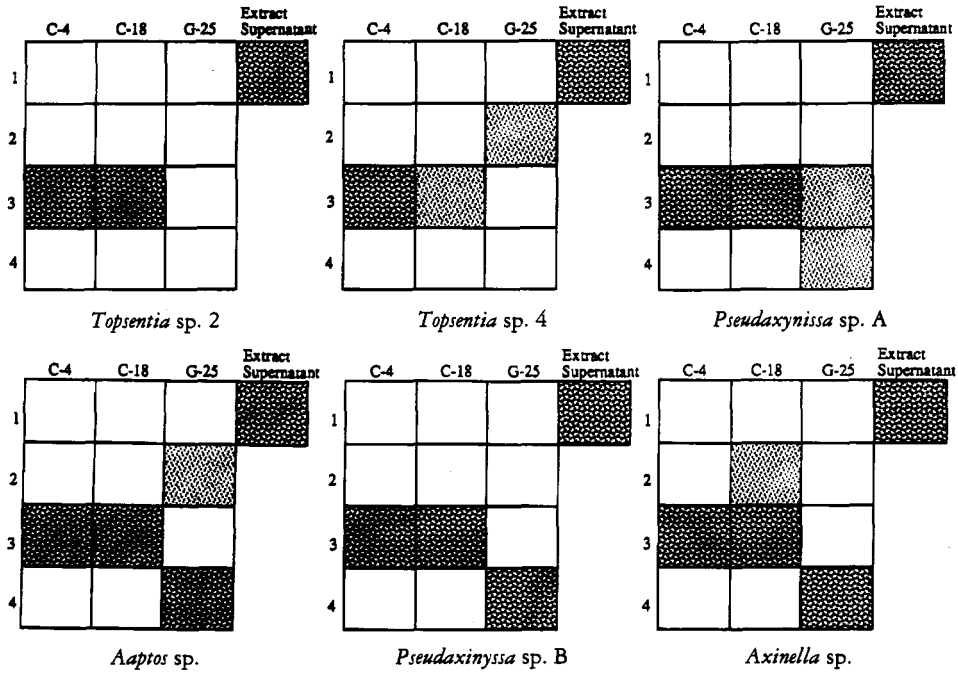
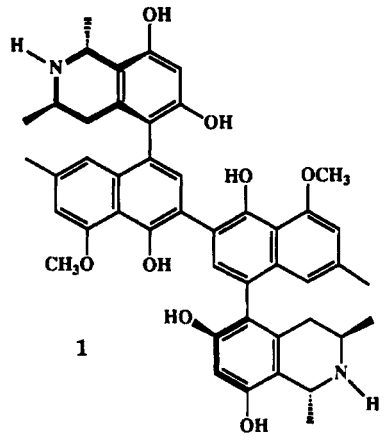


FIGURE 2. Chemical screening patterns for sponge extracts containing sulfated sterols. The 3×4 matrix represents four fractions each from three different chromatography media. Heavily shaded boxes indicate elution of anti-HIV activity comparable to a control sample of the extract supernatant. More lightly shaded boxes indicate elution of activity of lower potency than the control; open boxes indicate no elution of anti-HIV activity.

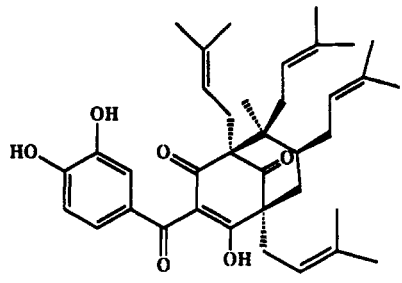
As noted earlier, the high rate of activity in supernatants of plant extracts was presumed to be due to tannins, and as anticipated, the polyamide cartridge has proved to be an enormously effective dereplication tool for extracts containing these compounds, eliminating over 90% (122 of 129 thus far tested; data not shown) of the supernatants from further consideration. To address concerns that other classes of HIV-inhibitory phenolic compounds might be irreversibly bound to the polyamide resin, we examined the behavior of michellamine B [**1**] (13, 19), peltatol A [**2**] (14), and guttiferone A [**3**] (15) on polyamide resin; all of these phenolic compounds eluted from the cartridges with complete recovery of HIV-inhibitory activity (data not shown). These results, together with the observations of Tan *et al.* (7), strongly suggested that only polyphenolic compounds will be irreversibly retained on polyamide gels.

The chemical screen protocol also pinpointed those supernatants in which activity remained due to incomplete precipitation of polysaccharides. Elution of anti-HIV activity in the first fraction from each cartridge or precipitation on the cartridge packings could be confirmed as indicative of anionic polysaccharides by evaluation of toluidine blue bathochromic shifts in the supernatants (16).

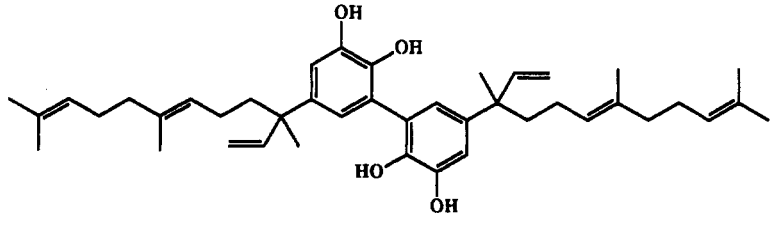
We have further utilized this chemical screening method in the preliminary analysis and prioritization of leads from among the organic extracts of plants. Our preliminary analysis of anti-HIV active organic extracts consists of a solvent-solvent partitioning scheme which produces hexane-, CCl_4-, $CHCl_3$-, EtOAc-, and H_2O-soluble fractions. We have found that a substantial portion (>60%) of the organic extracts of plants provide aqueous partition fractions that contain all or most of the HIV-inhibitory activity. Such aqueous partition fractions now routinely enter the chemical screening protocol at the polyamide cartridge (tannin dereplication) stage. This has proved to be



1



3



2

a very effective means of rapidly identifying and eliminating tannin-containing organic extracts from consideration for further chemical and biological analyses. The frequent appearance of tannins in plant organic extracts also explains the higher percentage of active organic extracts from plants versus all other taxa.

DISCUSSION

The chemical screen described above is simple, rapid, and relatively inexpensive. The method is versatile; other phases can be added or substituted to expand or alter the elution matrix or fingerprint. A good deal of information is gained at the cost of very small amounts of extract consumed. The protocol directly addresses the problem of dereplicating (eliminating from further consideration) extracts containing known, commonly occurring antiviral compounds, such as the anionic polysaccharides and tannins, and also provides a good starting point for the identification and facile follow-up dereplication of extracts containing other HIV-inhibitory agents that subsequently may prove also to represent a recurring class or classes of compounds.

It should be noted that a related concept of (and, in fact, the phrase) "chemical screening" has been used before. For example, in a series of articles published over the past decade, groups led by Hammann (17) and Zeeck (18) have described chemical screening for secondary metabolites by means of functional group- or compound-class-specific tlc detection reagents. Both groups describe their methods as independent of biological activity (17) or not taking biological activity into account (18). Such approaches can be very effective at pinpointing novel secondary metabolites and are employed with success by many natural product research groups worldwide. From our experience described herein, we conclude that our chemical screening protocol, in conjunction with our polysaccharide dereplication protocol, and coupled with the anti-HIV bioassay, constitutes an appropriate and efficient overall strategy for selection of

priority extracts for our subsequent bioassay-directed investigation of potentially novel active chemotypes.

EXPERIMENTAL

TANNIN DEREPLICATION.—Solid phase extraction columns were prepared by packing 1 g of Macherey Nagel polyamide SC6 resin, pre-swollen in H_2O, into a 12 cc polyethylene syringe fitted with a glass wool plug in the luer tip. The substrate to be analyzed (3–6 mg) was dissolved in a minimal amount of H_2O (<500 μl), applied to the column, and eluted in four fractions: 2 ml H_2O, 2 ml H_2O-MeOH (1:1), 5 ml MeOH, 5 ml MeOH. The fractions were evaporated, dissolved in 240 μl DMSO-H_2O (1:1), and tested for anti-HIV activity (see below).

CHEMICAL SCREEN.—Baker C_{18} and WPC_4 6 ml cartridges were used with an Analytichem Vac-Elut manifold. Cartridges were preconditioned for use by washing sequentially with three column volumes each of MeOH, MeOH-H_2O (2:1 and 1:2), and H_2O prior to use. The sample to be analyzed (3–6 mg) was dissolved in a minimal volume of H_2O (<500 μl) and applied to the column. Fractions of three column volumes each of H_2O, H_2O-MeOH (2:1, then 1:2), and MeOH were collected sequentially. The Sephadex G-25 cartridges were prepared by packing 1 g coarse grade gel, preswollen in ca. 6 ml H_2O, in a 6 ml polyethylene syringe; the gel was packed and rinsed by gravity flow with several column volumes of H_2O. As in the other cases, a 3–6 mg sample was dissolved in a small amount of H_2O (<500 μl) and applied to the column. Four fractions were collected, the first of 2 ml, then two of 750 μl, and a final fraction of 5 ml.

All column fractions were evaporated in vacuo and dissolved in 240 μl DMSO-H_2O (1:1) for anti-HIV testing (see below). To save time and effort, fractions were not weighed; the EC_{50} values generated from the primary anti-HIV screen assume full elution of the activity of the parent extract or supernatant in each fraction. Therefore, quantitative measurements of true EC_{50} values could not be made; qualitative assessment of activity recovery was gleaned from comparison of the activity found in fractions with that of the parent extract or fraction run as a control with the chemical screen fractions.

ANTI-HIV TESTING.—The anti-HIV assay was performed as described previously (2) with certain modifications. Briefly, the assay involves the killing of T4 (CD4+) lymphoid cells (CEM-SS line) by HIV-1 (RF strain) and the inhibition of this killing by active compounds, extracts, or fractions. The exponentially growing cells were pelleted from the growth medium, infected at a multiplicity of infection (MOI) of 0.05 at room temperature for 45 min with constant agitation, and diluted in growth medium to the desired cell concentrations to yield 5000 cells/well after inoculation and appropriate dilutions into individual wells of 96-well microtiter plates. Equal aliquots (50 μl) of the DMSO-H_2O test solutions were added to the appropriate wells, and the plates were incubated for 6 days at 37°. Plates were then analyzed for cellular viability using the XTT-tetrazolium method as described (2).

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