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Frequent occurrence of HIV-inhibitory sulphated polysaccharides in marine invertebrates

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Summary

Aqueous extracts of many marine invertebrates have exhibited some activity in the National Cancer Institute's primary screen for anti-HIV cytopathicity. Using a variety of techniques, including gel permeation, size exclusion and ion exchange chromatography, toluidine blue metachromicity, ^{13}C-NMR spectroscopy and combustion analyses, we have determined that this s activity is largely due to sulphated polysaccharides. Because of the wide occurrence of this class of compounds in these organisms we sought a method a for the rapid dereplication of sulphated polysaccharides. It was critical that the method selected for dereplication allow differentiation of anionic polysaccharides from other AIDS-antiviral chemotypes. After evaluating a variety of methods, we found that the most efficient strategy appeared to be precipitation of the polysaccharide fraction from aqueous ethanolic solutions of the crude aqueous extracts.

Introduction

Organic and aqueous extracts from a wide variety of marine invertebrates, terrestrial plants and microorganisms are being tested for anti-HIV activity in the NCI's large-scale screening programme (Boyd, 1988; Weislow et *al.,* 1989). A disproportionately high percentage (23%) of the crude aqueous extracts of marine invertebrates has shown some activity in the primary AIDS-antiviral screen. Many such extracts have displayed bioactivity profiles similar to dextran sulphate and other sulphated polysaccharides. Biopolymers of this class have been reported previously from diverse marine invertebrates (e.g., Katzman and Jeanloz, 1970; Humphreys *et al.,* 1977; Junqua *et al.,* 1979; Albano and Mourao, 1983, Coombe *et al.,* 1987; Valiente *et al.,* 1988) but have not previously been shown to have anti-HIV or other antiviral activity from such organisms.

In contrast, polysaccharides from marine algae have long been associated with antiviral activity. In 1958, Gerber *et al.,* reported that algal polysaccharides exhi-Jited antiviral activity towards mumps and influenza B liruses. Ehresmann *et al.* (1977) linked the inhibition of lerpes simplex and other viruses with polysaccharide fractions from extracts of ten red algae; similar observations were made by Richards *et al.* (1978). More recently, Nakashima *et al.* (1987a, 1987b), have characterized the HIV reverse transcriptase inhibitory sulphated polysacharide from the red alga *Schizymenia pacifica.*

In a comparative evaluation of a diverse selection of sulphated polysaccharides, Baba *et al.* (1988), found that many, but not all, had anti-HIV activity. Sulphated homo- polysaccharides have been reported generally more active than sulphated heteropolysaccharides, while the glycosaminoglycans, such as chondroitin sulphates A and B, have been found inactive (Mizumoto *et al.,* 1988). More recently, fucoidan, a complex sulphated polysaccharide from the alga *Fucus vesiculosus,* was found to inhibit HIV *in vitro* and was synergistic with AZT. This activity presumably was due to a direct interaction of the polysaccharide with the HIV binding site on the membrane of target cells (Suguwara *etal.,* 1989).

We undertook the present study of the sulphated polysaccharides from marine invertebrates to isolate and characterize representative polysaccharides and to develop protocols for the rapid, efficient dereplication of this class of compounds. It was essential that we identify a method which would not only indicate the presence of anionic polysaccharides, but also allow differentiation of those biopolymers from any other AIDS-antiviral chemotypes.

Results

Polysaccharides in the crude aqueous extract (2g) of *Galaxea sp.* (Cnidaria, EC50 22 μg ml-1) were

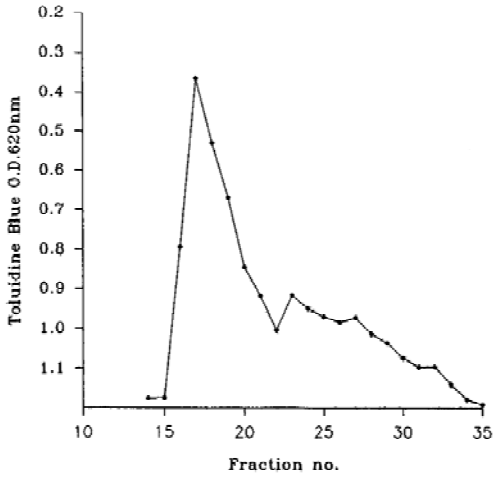

Fig. 1. Size-exclusion chromatography on Sephadex G-25 (2 x 94cm) of EtOH precipitate of *Galaxea sp.* aqueous extract. Toluidine blue dye binding values for the fractions are given on the Y axis.

precipitated from water by addition of an equal volume of EtOH, yielding a precipitate (257 mg) with an anti-HIV EC_{50} of 5 μg ml-1. The lyophilized supernatant was partitioned between nBuOH and H_2O; the smaller nBuOH-soluble fraction was modestly cytotoxic to the lymphocytes used in the anti-HIV assay (IC_{50} 77 μg ml-1), but provided no protection against the cytopathic effects of HIV; the water fraction was entirely inactive at 100 μg ml-1.

The HIV-inhibitory precipitate was fractionated on a Sephadex G-25 size exclusion column eluted with water (Fig. 1). The first fraction (44mg) was protective against HIV at 3 μg ml-1, while subsequent fractions had progressively less activity. Ion exchange chromatography of the high molecular weight fraction on DEAE cellulose with a linear NaCI gradient eluted 31 mg of anionic polysaccharide with an EC_{50} of 2.6 μg ml-1 (Fig. 2). This material was examined by ^{13}C-NMR in D_20 at 70°C (Fig. 3). Important features in the spectrum included a methyl resonance at 16 p.p.m., several anomeric carbons around 104 p.p.m., numerous oxygenated methine and methylene resonances between 60 and 83 p.p.m. and the absence of carbonyl peaks. Taken together, these indicated a polysaccharide with modest 6-deoxy-sugar content, no N- acetyl or O-acetyl functionality, and an absence of the anhydrogalactose unit found in the agarose series of polysaccharides. The bioactivity of the sample was undiminished after 14 h at 70°C.

High performance size exclusion chromatography (SEC) was used to estimate the molecular weight of the anti-HIV active polysaccharide. While elution with water gave anomalous calibrations due to ionic exclusion of the negatively charged polysaccharides, elution with 0.1 M $NaNO_3$ gave acceptable calibrations with standard materials. The polysaccharide fractions from four invertebrates *(Galaxea sp., Reniochalina stalagmitis, Pyrosoma sp.,* and *Sigillina sp.)* were examined by this method and were all found to have molecular weight averages between 500 and 1000 kD (Fig. 4).

Similar fractionation of aqueous extracts of the sponges *Reniochalina stalagmitis, Ircinia felix* and *Niphates digitalis,* a deep-water tunicate, *Pyrosoma sp.,* a shallow water tunicate *Sigillina sp.,* as well as the cnidarian *Montastrea curta* gave very similar results, namely anti-HIV polysaccharides which were characterized in the same fashion as those from *Galaxea.*

In the case of *Reniochalina stalagmitis* (Porifera), combustion analysis of the active materials revealed sulphur content consistent with one sulphate group per 3-4 sugar moieties. A sample measured before ion exchange showed a high analysis for nitrogen (7.5%), but this percentage dropped substantially after ion exchange, indicating either that a counterion contained nitrogen (e.g., guanidinium or ammonium) or that there was a substantial amount of protein contaminating the preparation.

Combustion analyses require rather large masses of material. Therefore, with polysaccharide fractions from other extracts, IR spectroscopy was considered as an alternative to estimate the degree of sulphation. This technique has been used for carrageenan and agar samples *(*Rochas *et al.,* 1986); it involves comparison of the IR absorbance of films at two absorbance bands, one (2920 cm^{-1}, C-H) representing total sugar content, and the other (1250 cm-1) representing total

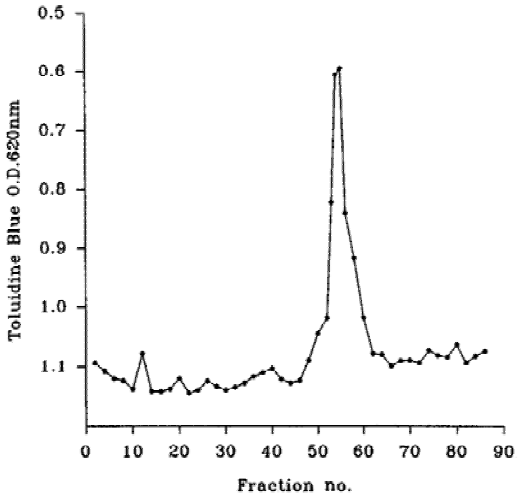

Fig. 2. DEAE Cellulose ion-exchange chromatography of high molecular weight fraction from Sephadex G-25 chromatography of *Ga/axea sp.*

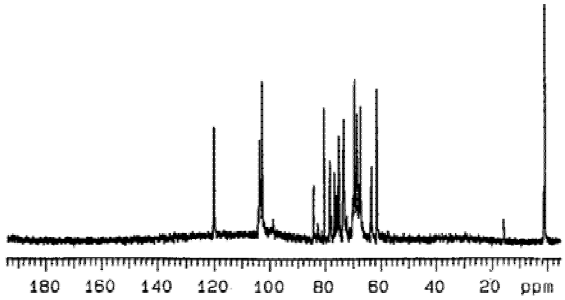

Fig. 3. 13C-NMR sectrum of active *Galaxea sp.* fraction from DEAE ion exchange chromatography. Peaks at 120p.p.m. and 3p.p.m. are due to acetonitrile as chemical shift reference. See experimental section for acquisition conditions.

sulphate. Without exception, all of the polysaccharide fractions showed sulphate bands in the vicinity of 1250 cm^{-1}, although careful control of water content was required to resolve the band at 2920 cm^{-1} from the broad H_2O band. The 1250 cm^{-1} band was also broad, and several of the polysaccharides examined did not give films suitable for analysis.

Albano and Mourao (1983, 1986) have demonstrated the use of the cationic dye toluidine blue to estimate the concentration of polyanionic molecules in aqueous solution and applied the method to the determination of sulphated polysaccharides in sea cucumbers and tunicates. We measured the toluidine blue metachromatic shifts of crude extracts and their EtOH precipitates for 28 different organisms from a variety of taxa (see Materials and Experimental procedures). These values were com pared to the EC_{50} values from the anti-HIV assay. It appeared that these points were largely clustered in a linear array. Five points which fell furthest from this cluster were not included in a linear regression analysis (Fig. 5, crude extract data). We presumed that these points represented samples in which the anti-HIV activity was not due only to the sulphated polysaccharide content of the extract. The points along the regression line represent cases where sulphated polysaccharide content is the primary reason for the bioactivity of these samples. In support of this interpretation, it is noteworthy that extrapolation of the calculated regression line to an EC_{50} of ca. 0.1 $\mu g\ ml^{-1}$, which is close to the value we observe for pure sulphated polysaccharides vs. HIV, gives a polysaccharide concentration near unity. Samples which fell furthest from the regression line were of greatest interest (Fig. 5). While there was an overall correlation between toluidine blue metachromatic shift and anti-HIV potency, it was not consistent enough to justify using only the toluidine blue value of the crude extract or precipitate as a dereplication criterion. Colour interferences in the crude extracts gave erroneous results in a significant number of cases.

Discussion

Based on the comparisons reported above, the *Galaxea, Reniochalina,* and *Pyrosoma* polysaccharides which were characterized in detail all appeared to be sulphated fucans, similar to, but distinct from fucoidan, a known active material. The difference included a lesser degree of branching than fucoidan, as judged from the relatively simple ^{13}C-NMR spectra. Although characterized in less detail, the polysaccharides from the other organisms appeared similar.

The definitive sources and functions of these sulphated polysaccharides in invertebrates are not entirely clear. Interestingly, sponge aggregation factors (Humphreys *et al.,* 1977) are thought to consist of proteins crosslinked through Ca^{+2} to sulphated polysaccharides. Similarly, Coombe *et al.* (1987), have proposed a role for sulphated polysaccharides in species-specific sponge cell aggregation. The sheaths, or tunics, of the ascidians (tunicates) are thought to be comprised largely of sulphated polysaccharides. Also, the resemblance to cartilage constituents is great, although invertebrate polysaccharides are distinguished by their lack of N-acetyl groups. They may play a role in the structural integrity of the organism (Katzman and Jeanloz, 1970).

Anti-HIV activity has been detected in at least some representatives of all marine phyla which we have examined to date (Porifera, Chordata, Crustacea, Bryozoa, Annelida, Echinodermata, Mollusca, and Cnidaria). Given the presumed ubiquitous presence of sulphated polysaccharides and related substances in

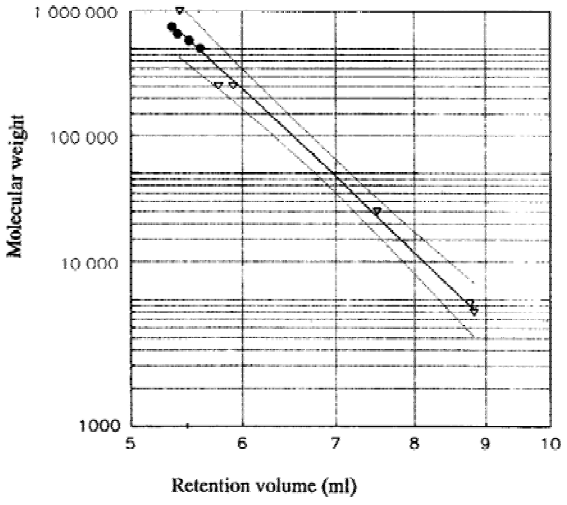

Fig.4. High performance size exclusion chromatography of sulphated polysaccharide standards and isolated invertebrate sulphated polysaccharides. Open triangles in descending order of molecular weight, blue dextran (2000kD), dextran sulphate (500kD, two peaks), chondroitin sulphate A (50kD), dextran (9.4kD), dextran sulphate (8kD). Closed circles represent active polysaccharide fractions from *Galaxea sp., Reniochalina stalagmitis, Sigiflina sp., Pyrosoma sp.* A., also in descending order of molecular weight. Conditions: TosoHaas G3000PW column eluted with 1 ml min-1 0.1 M $NaNO_3$.

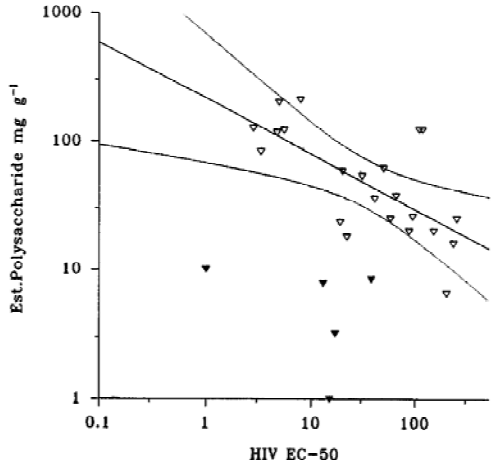

Fig. 5. Log-log plot of HIV ECso values for 28 crude marine aqueous extracts vs. toluidine blue dye binding values. The solid line represents a regression line generated by a least squares fit of the data points; points represented by solid triangles (A) were omitted from the calculation. The dotted lines represent 95% confidence levels of the calculation. Extracts represented by data points below the dotted line would be candidates for further investigation.

marine invertebrates, it is perhaps surprising that an even greater percentage of these extracts do not show anti-HIV activity in the primary screen.

We suggest some additional insight in this respect from the parallel examination of two collections of the tunicate genus *Sigillina.* Aqueous extracts from one were protective in the anti-HIV assay at 4 μg ml^{-1}, while similar extracts of the other were entirely inactive at 200 μg ml^{-1}. The active *Sigillina* constituent was characterized as a sulphated polysaccharide, while the inactive crude extract yielded only a weakly active EtOH precipitate. While this might suggest that these polymers are not ubiquitous constituents of marine invertebrates, even within species, it is also possible that our extraction methodology produces quite variable yields of polysaccharides. Alternatively, different structural classes of sulphated polysaccharides may have different levels of anti-HIV activity, or certain types of polysaccharide may have poor stability under the extraction conditions used.

The observed shortcomings and inconsistencies of the size-exclusion HPLC and IR analyses rendered them unsuitable as dereplication tools.. While the gel permeation, ion exchange and ^{13}C NMR analyses gave useful and reproducible data, the experiments were too tedious and time-consuming for application to the dereplication of hundreds of leads. As noted above, the toluidine blue method offered the desired characteristics of speed and simplicity, but measurements with both crude extracts and precipitates apparently suffered from interference by other anionic components and/or pigments in the extracts. Ultrafiltration was also evaluated as a means of prioritizing leads on the basis of molecular size and weight, but the flow rates with crude extracts were unacceptably slow and decomposition and diminution of activity was observed in a number of cases.

In contrast, the simple precipitation experiment used in the fractionation protocol with the *Galaxea* extract was rapid, simple and efficient in confining all the bioactivity in the precipitate fraction. Application of this procedure to all of the extracts found to contain antiviral polysaccharides by means of the more detailed analyses described herein located all the HIV-inhibitory activity in the precipitates.

Consequently, we have adopted this precipitation methodology for the preliminary evaluation of all crude aqueous extracts of marine invertebrates and the dereplication of sulphated polysaccharides. To date, 531 aqueous extracts of marine invertebrates have been subjected to the EtOH precipitation; only 64 of the supernatants (12.1 %) exhibited any HIV-inhibitory activity (see Table 1). All the active supernatants were confined to the Porifera, Echinodermata, Cnidaria and Chordata. Perhaps the most notable result was that all the antiviral activity in all but three of the 80 tunicate extracts examined was found solely in the precipitates. While sulphated polysaccharides have attracted considerable attention as potent inhibitors of HIV-1 *in vitro,* our primary interest is to identify entirely new chemotypes with anti-HIV activity. Therefore, the aqueous marine extracts which yield bioactive supernatants in this protocol (i.e. indicating the presence of anti-HIV activities not due to sulphated polysaccharides) are the priority focus for our continuing detailed chemical and biological study.

Table 1. Summary of precipitation protocol results

Extracts Pptd	Phylum #	active supernatants
Annelida	6	0
Bryozoa	6	0
Chordata	80	3
Cnidaria	98	9
Crustacea	16	0
Echinodermata	65	10
Mollusca	27	0
Porifera	233	42
Total	531	64

Materials and Experimental procedures

Marine organisms

All organisms are documented by preserved voucher specimens deposited at the Smithsonian Oceanographic Sorting Center, Suitland, MD. Voucher collector numbers are listed below.

(1) *Galaxea sp.* (Family Oculinidae, Order Scleractinia, Class Zooantharia, Phylum Cnidaria) was collected in January, 1987, encrusting a rock/coral cliff wall at 6 m by SCUBA at Little Broadhurst reef, on the Great Barrier Reef of Australia, voucher specimen Q66CO031, identified by Russell Kelley.

(2) *Montastrea curta* (Family Faviidae, Order Scleractinia, Class Zooantharia, Phylum Cnidaria) was collected in January, 1987, at 1.5 m on a coral ridge at Little Broadhurst reef, on the Great Barrier Reef of Australia, voucher Q66CO042, identified by Terry Done.

(3) *Reniochalina stalagmitis* (Family Axinellidae, Order Axinellida, Class Demospongiae, Phylum Porifera) was collected by SCUBA at 9m, growing on a rock substrate in Darwin harbor, Darwin, Australia, in August, 1987, voucher specimen Q66C0574, identified by John Hooper. (4) *Ircinia felix* (Family Spongiidae, Order Dictyoceratida, Class Demospongiae, Phylum Porifera) was collected by submersible at 90 to 140m depth in the Bahamas in March, 1987, voucher Q66B0932, identified by Shirley Pomponi.

(5) *Niphates digitalis* (Family Niphatidae, Phylum Porifera) was collected in June, 1988, off Acklins Island, Bahamas by SCUBA, vouchers Q6711271, Q6711272 and Q6711276, identified by Harley Sheffield.

(6) *Pyrosoma sp.* (Order Pyrosomida, Class Thaliacea, Phy1um Chordata) was collected in December, 1986, in the Galapagos Islands at a depth of 548 m by submersible, voucher Q66A0330.

(7) *Sigillina sp.* (Family Holozoidae, Order Aplousobranchia, Class Ascidiacea, Phylum Chordata) was collected encrusting a dead animal shell at 20m on the Parry Shoals, west of Bathhurst Island, Australia, in August, 1987, Q66C0524.

(8) *Sigillina sp.* (Family Holozoidae, Order Aplousobranchia, Class Ascidiacea, Phylum Chordata) was collected at 8.5 m growing as a colony on a rock substrate, at Arrawarra on the Australian coast, in February, 1988, identified by Rob McCauley.

The following twenty-eight organisms were used to evaluate the relationship between HIV EC_{50} and toluidine blue values:

Chordata Tunicata: *Polysyncraton sp.* (Voucher Q66C0783), *Polycarpa pigmentara* (Voucher Q66B2171), *Pseudodistoma sp.* (Voucher Q66C0828), *Pyura gibosa gibosa* (Voucher Q66C0940).

Cnidaria: *Agaricia agaricites* (Voucher Q66B0424), *Antipathes sp.* (Voucher Q66C0705), *Cespitularia sp.* (Voucher Q66C0237), *Diploria strigosa* (Voucher Q66B0575), *Efflatounaria sp.* (Voucher Q66C0854), *Goniastrea edwardsi* (Voucher Q66CO038), *P!atygyra pini* (Voucher Q66CO047), *Plexaura flava* (Voucher Q66C0146), *Solenocaulon sp.* (Voucher Q66C0745).

Echinodermata: *Holothuria leucospilota* (Voucher Q66C0760), *Stichopus horrens* (Voucher Q66C0761).

Mollusca: *Australium tentoriforme* (Voucher Q66C0925), *Bembicium nanum* (Voucher Q66C1 012).

Porifera: *Amphimedon sp.* (Voucher Q66C0466), *Arenosclera sp.* (Voucher Q66C0836), *Cinachyra sp.* (Voucher Q66B1064), *Cinachyra sp.* (Voucher Q66C0447), *Clathria (Dendrocia) pyramida* (Voucher Q66C0980), *Dragmaxia sp.* (Voucher Q66B1 060), *Iotrochota sp.* NTM 361 (Voucher Q66C0727), *Niphates erecta* (Voucher Q66B0331), *Pseudaxynissa sp.* NTM 664 (Voucher Q66B2242, Q66C0816), *Verongula sp.* (Voucher Q66B1092).

Chemistry

Precipitation

Aqueous extracts were prepared by the method of T.M. McCloud et *al.* (personal communication), from frozen marine organisms. The lyophilized extracts were dissolved in distilled water at 50 mg ml^{-1} and precipitated with an equal volume of EtOH (-20°C, overnight), centrifuged at 1000 r.p.m., and decanted. The EtOH was removed from the supernatant by rotary evaporation or Speedy-Vac and both fractions were then freeze-dried.

Size exclusion chromatography

Extract samples (250 mg) were subjected to size exclusion chromatography utilizing Sephadex G-25 (Sigma) columns (0.9 x 24cm) eluted by gravity with H2O at ambient temperature. High-performance Size-Exclusion Chromatography (SEC) employed TosoHaas TSK-gel columns, G3000PW and G4000PW, 7.5mm x 30cm, eluting with 0.1 M $NaN0_3$ at 0.5ml min^{-1} from a Waters 600E pumping system equipped with a model 401 refractive index detector.

Ion-exchange chromatography

Ion-exchange chromatography was performed using either DEAE Cellulose (Sigma) in a 2.5 x 22 cm glass column, or a DEA MemSep 1010 Chromatography cartridge (Millipore), and a Fluid Metering, Inc. model OSY pump. Initial buffer was 50 mM NaCl, in 10 mM tris-HCI (pH 7.0). After loading, a linear gradient was formed using a gradient mixer (Bethesda Research Labs) from initial buffer to 1.5 M NaCI in 10 mM tris HCI (pH 7.0). The gradient volume was 300-600 ml. Fractions were combined based on toluidine blue metachromatic shifts, freeze-dried, then desalted by ultrafiltration using a Novacell5 kD disposable cell (Filtron), and freeze-dried.

Toluidine blue (Albano and Mourao, 1986)

An aqueous solution of Toluidine Blue 0 (C.I. 52040, Sigma) was made at 10 μg ml^{-1} in H_2O. Aliquots of fucoidan were added to disposable polystyrene cuvettes, then 1 ml of toluidine blue solution was added to each. The solutions were allowed to stand for 10 min. Then the absorbance of each was measured at 620 nm to construct a standard curve. Dye binding induced a metachromatic shift in wavelength which resulted in a decrease in absorbance with increasing sulphated polysaccharide concentration. This relationship was linear between 1 and 10 μg of fucoidan. Aliquots of chromatographic fractions (10-200 μl) were assayed in the same fashion, and the absorbance values were used to plot synthetic chromatograms (e.g., Figs 1 and 2).

^{13}C-NMR

Samples were dissolved in D_2O at concentrations of >20mg ml^{-1}, and examined at 125 MHz using a Varian VXR-500 spectrometer equipped with a broadband probe. The temperature was 70°C, acquisition time

0.2 s, pulse width 7.5 ms, delay 0. Ca. 250,000 transients were required to give adequate S/N. Acetonitrile (25 μl) was used for chemical shift reference.

Infrared films (Rochas *et al.,* 1986)

Films for infrared spectroscopy were prepared by evaporating 2ml of a 10 mg ml^{-1} solution of the material in H_2O in teflon moulds at 40°C in an oven. The film was mounted between two NaCI plates and the IR spectrum measured with a Perkin-Elmer model 1600 fourier transform infrared spectrometer.

Anti-HIV testing

The XTT-tetrazolium anti-HIV assay was performed on crude extracts, chromatographic fractions, and pure compounds as previously described (Weislow et *al.,* 1989). Confirmatory assays were performed as reported elsewhere (Gulakowski et *al.,* 1991).

Acknowledgements

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